The role of serpins and granzymes in cancer and immunity

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The role of serpins and granzymes in cancer and immunity

De rol van serpins en granzymen
in kanker en immuniteit

(met een samenvatting in het Nederlands)

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General introduction

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The immune system at a glance

The immune system protects our body against pathogens, like viruses and bacteria, as well as abnormal cells, *e.g.* damaged and transformed cells. The first line of defense is provided by cells of the innate immune system, consisting of neutrophils, eosinophils, basophils, mast cells, natural killer (NK) cells, dendritic cells (DC), and macrophages. Most of these cell types express receptors that recognize conserved pathogen-associated molecular patterns (*e.g.* LPS) or stress signals in a non-specific way [1]. Upon identification of a micro-organism or an abnormal cell in the body, cells of the innate immune system rapidly eliminate their targets via phagocytosis or secretion of toxic granules [2]. In addition, cells of the innate immune system can initiate an adaptive immune response.

Lymphocytes, *i.e.* B cells and T cells, of the adaptive immune system provide a delayed but highly specific defense mechanism, including increased protection upon re-infection. An adaptive immune response is initiated by antigen presenting cells (APC), *e.g.* dendritic cells. APC continuously present peptides (antigens) from endogenous proteins on their cell membrane, and in case of infection also from the pathogen [3]. T cells that express a highly diversified receptor specifically recognize such an antigen and are activated by the APC in lymphoid tissues, resulting in clonal expansion of the T cells [4]. APC use two types of major histocompatibility complex (MHC) molecules to present antigens to T cells [5]. MHC class I molecules present endogenous and foreign peptides from the cytoplasmic compartment of the APC and can activate $CD3^+/CD8^+$ cytotoxic T lymphocytes (CTL), resulting in a cellular cytotoxicity response. MHC class II molecules present peptides from the vesicular compartment, including internalized proteins, which can lead to macrophage or B cell activation via $CD3^+/CD4^+$ T helper lymphocytes. Activated B cells on their turn secrete antibodies and induce a humoral immune response. In both cases, the T cell-mediated response results in the elimination of pathogens or abnormal cells.

So, activation of cells of the innate and/or adaptive immune system by pathogens and abnormal cells finally lead to elimination of the latter ones via phagocytosis, an antibody-mediated humoral response, or cellular cytotoxicity. The first part of this thesis focuses on the cytotoxic proteases expressed by the killer cells of the immune system.

Cytotoxic lymphocytes

The major players in the cellular immune response against virus-infected and tumor cells are the cytotoxic lymphocytes (CL), *i.e.* CTL and NK cells [6]. Both these cell types are very potent inducers of cell death, but they recognize their target cells in a different way. CTL are initially activated in lymphoid organs upon encountering of a T cell-receptor-specific antigen presented by APC using MHC class I along with a co-stimulatory signal [5]. After clonal expansion, CTL can recognize the same antigen–MHC-complex presented on the surface of peripheral virus-infected or tumor cells, and subsequently attack their target cells.

In contrast, NK cells act without prior sensitization and their function is regulated by the balance of multiple inhibitory and activating receptors, *e.g.* members of the NKG2 and KIR families [7]. A major mechanism that prevents activation of NK cells is binding of their inhibitory receptors to MHC class I molecules presenting self-peptides. Target cells that lack or have low expression of MHC class I molecules initiate a reduced signaling via these inhibitory receptors resulting in NK cell activation; a process named ‘missing self’ hypothesis

[8]. NK cells can be distinguished by the expression of CD16 and CD56 on their cell membrane, while CD3 is absent. It should be noted that there are also two small populations of cells with combined characteristics of both CTL and NK cells, named Natural Killer T (NKT) cells and γδ T cells [9,10]. Thus, the mechanisms of target cell recognition and subsequent activation are different between CTL and NK cells. Nevertheless, these two major types of killer cells induce cell death via similar pathways.

Two cell death pathways: death receptor and granule-exocytosis

Upon recognition of a target cell, cytotoxic lymphocytes can induce cell death via two mechanisms: the death receptor pathway and the granule-exocytosis pathway [11] (Figure 1). In the first pathway, CTL and NK cells express death receptor-ligands (*e.g.* FASL) on their plasma membrane. Upon engagement of such a ligand with its accessory receptor that is expressed by a target cell, the death receptor is stimulated and recruits the initiator caspases, pro-caspase-8 and -10, via interaction with the converging death receptor adaptor protein FAS-associated death domain (FADD) [6]. The resulting active caspase-8 and -10, on their turn, can initiate the release of mitochondrial cytochrome c via cleavage of the pro-apoptotic BCL-2 family member BID, or directly cleave pro-caspase-3. Both actions lead to activation of the executer caspases, caspase-3, -6, and-7, which finally induce cell death by DNA fragmentation [12].

The second mechanism to induce cell death is via the granule-exocytosis pathway. In this case, activated cytotoxic lymphocytes release the content of their pre-formed granules into the immunological synapse between the effector and target cell [13]. These secretory vesicles contain the key players to induce cell death in a target cell, *i.e.* the pore-forming protein perforin and a family of serine proteases known as granzymes (granule-associated enzymes). While perforin facilitates the entry of the granzymes into a target cell, granzymes cleave specific intracellular substrates, thereby initiate cell death routes in a target cell [14]. As granzymes are the main topic of the first part of this thesis, the components of the granule-exocytosis pathway will be described below in more detail.

Perforin-dependent entry of granzymes into a target cell

Perforin is a pore-forming protein that facilitates the entry of granzymes into a target cell (Figure 1). The membrane permeabilization capacity of perforin is crucial for an effective granule-exocytosis mediated cell death [15]; however, the underlying mechanism has been undergoing a renaissance lately. After secretion into the immunological synapse, perforin molecules multimerize in the plasma membrane of target cells, thereby forming transient pores [16-18]. As these holes are likely too small for single granzymes proteins to pass efficiently, two alternative models have been suggested, although caution should be taken because the perforin concentrations used *in vitro* might not represent the situation *in vivo* [19,20]. First, together with perforin, granzymes have been proposed to be endocytosed by target cells in a perforin-independent way. Granzymes are then released into the cytoplasm by perforin-mediated lyses of the endosomes [21,22]. Indeed, GrB is able to bind to specific membrane receptors by charge, although these receptors appear not crucial for endocytosis of the cytotoxic protease [23-25]. Second, a rapid membrane repair-response upon a perforin-induced membrane calcium flux has been suggested, resulting in internalization of membrane-bound granzyme molecules. Whether large endosomes and a second action of perforin are involved in this model remains to be elucidated [26,27].

Granzyme-mediated cell death

Granzymes are serine proteases that can induce cell death upon entry of a target cell (Figure 1). Intracellular, both in the cytoplasm and the nucleus, they can cleave specific substrates, *e.g.* pro-caspase-3 by GrB, thereby initiating multiple cell death pathways [13]. As each granzyme has its own preference for a set of substrate proteins, they induce cell death via unique as well as overlapping mechanisms, with corresponding morphological hallmarks of target cells [14]. The characteristics and specificities of the human granzymes will be discussed in the next sections.

Characteristics of the human granzymes

The human family of granzymes consists of 5 members: GrA, GrB, GrH, GrK, and GrM [28] (Table 1). This non-sequential nomenclature of the human granzymes is a result of the large amount of especially murine granzymes, which lack a human equivalent, *e.g.* GrC-G. The less-diversified human granzymes all have murine orthologues, except for GrH. However, evidence for differences in substrate-specificity between the human and murine orthologue granzymes is emerging recently [29-32], which make granzyme knock-out mouse models difficult to extrapolate to men. This thesis focuses on several members of the human granzyme family. Granzymes are initially synthesized by cytotoxic lymphocytes as zymogens (pre-pro-enzymes) and transported to the secretory lysosomes. In these granules, the granzymes are processed twice into fully mature cytotoxic proteases by enzymes like cathepsin C [33,34]. This results in active granzymes with a molecular weight of around 28 kDa, except for GrA that can form homodimers [35]. In addition, granzymes can undergo N-linked glycosylation.

The human granzymes are homologous in their amino acid sequences for about 40%, therefore similar in structure, and share the catalytic triad residues His^{57}, Asp^{102}, and Ser^{195} [28]. Using this catalytic triad, granzymes can cleave their substrates like most enzymes. Cristal structures of human GrA and GrB clarified that the substrate binding pocket largely determines their specificity [36-38] (Figure 2).

Based on their distinct chromosomal localizations, the human granzymes indeed display differences in their substrate specificity (Tabel 1). GrA and GrK are clustered on chromosome 5 and both demonstrate a tryptase-like activity, *i.e.* substrate-cleavage after Arg and Lys residues [39,40]. GrB and GrH reside at the chymase-locus on chromosome 14 and harbor a preferable specificity for Asp and Phe residues, respectively [41]. GrM is encoded by the neutrophil elastase gene cluster on chromosome 19, with Met and Leu as primary target residues in substrates [42]. Whereas GrA and GrB have been studied extensively, the elucidation of the characteristics of the three orphan human granzymes only started some years ago [43].

Upon binding of a specific substrate, granzymes hydrolyze a peptide bond in such target proteins. This mechanism of substrate cleavage is similar for most serine proteases, including the granzymes. The specificity of the serine protease-substrate interaction is mainly determined by substrate recognition sites in the protease, *e.g.* residues in the substrate binding pocket, which form hydrogen bonds with amino acids of a substrate [44]. The polypeptide binding site of the protease is numbered S3-S2-S1-S1'-S2'-S3' and match with residues in the substrate (P3---P3'), of which the S1-P1 interaction is of most importance for the specificity [45] (Figure 2). A bound substrate is mainly attacked by Ser^{195} of the catalytic triad of a serine protease, with assistance from His^{57}. Several intermediate structures are then formed as a result of diverse hydrogen bonds between the protease and target peptide. Finally, the amino bond between the P1 and P1' residue of the substrate is hydrolyzed, resulting in two cleavage-

products that are released by the protease, which remains active [46]. Biochemical studies for serine proteases often include an inactive mutant, in which Ser^{195} of the catalytic triad is mutated into an Ala ($S^{195}A$).

Granzyme substrates and the onset of target cell death

The major function of the granzymes is rapid induction of cell death by cleavage of specific intracellular substrates. As the preferred residues for cleavage are different for each human granzyme, they all hydrolyze unique substrates, but also overlapping target proteins at different sites. Cleavage of key proteins results in target cell death via distinct pathways in a few hours, of which most substrates converge into several major effector routes, *i.e.* mitochondrial and DNA damage via caspase-dependent and -independent ways [14,47]. Here, important substrates for each human granzyme as well as their corresponding cell death routes will be explained (Table 1).

GrA mainly induces cell death via mitochondrial damage and single-stranded DNA nicks. The mitochondria are important in the initial phase of GrA-induced cell death as GrA first induces loss of mitochondrial inner membrane potential via cleavage of the NDUFS3 subunit a oxidoreductase complex, resulting in generation and release of reactive oxygen species (ROS) [48-50]. The latter one drives the critical ER-associated SET complex, involved in DNA repair and consisting of the proteins SET, Ape1, pp32, HMG2, NM23-H1, and TREX1, into the nucleus. Nuclear-translocated GrA then cleaves SET, Ape1, and HMG2, resulting in inactivation of the DNA repair mechanism and, moreover, release of the nucleases NM21-H1 and TREX1 [51-53]. This finally leads to single-stranded DNA nicks and causes cell death. In addition, GrA cleaves proteins that are involved in the maintenance of chromatin and nuclear structure, or recognition of DNA damage, i.d. histone H1, lamins, and Ku70, respectively [54-56]. The concept of human GrA-induced cell death was recently challenged as it only appeared at very high concentrations (µM range), although the local granzyme concentration after delivery into a target cell via immunological synapses remains unknown [31,57,58].

GrB-mediated cell death follows the classical apoptosis pathway, including caspase activation and mitochondrial damage, resulting in DNA fragmentation. As GrB is by far the most studied one of the five human granzymes, many GrB-substrates have been identified. GrB can active the caspase route both directly, by cleavage of caspase-3, -7, -8, and -10, and indirectly, in a mitochondrial-dependent way [59-61]. In addition, GrB can also directly cleave (key) caspase-substrates, like ICAD, DNA-PK, and NuMA [62,63]. A critical GrB substrate in the cytoplasm is Bid. Together with the pro-apoptotic BCL-2 family members BAK and BAX, truncated Bid (tBid) permeabilizes the mitochondrial outer membrane, resulting in release of chytochrome c into the cytoplasm [64-66]. There, cytochrome c facilitates the formation of a complex between APAF-1 and pro-caspase-9 molecules, known as the apoptosome, which subsequently initiates a caspase cascade via activated caspase-9 [67]. This strengthens the caspase cascade that is also directly initiated by GrB. The final DNA damage is mainly mediated by GrB-dependent proteolytic cleavage of the inhibitor of caspase-activated DNase (ICAD), resulting in active CAD [63,68]. Translocation of the latter one into the nucleus leads to DNA fragmentation. This route of GrB-induced cell death is powerful, as cleavage of ICAD occurs both directly by GrB itself and indirectly by the GrB-activated executioner caspase-3. In addition, a variety of direct GrB-substrates have been identified that normally fulfill pro-survival functions, such as α-tubulin, ROCK-II, and lamin B (maintenance of cellular or nuclear integrity), PARP (DNA damage sensor enzyme), and MCL-1 (BCL-2 family

member) [54,69-73]. However, the physiological relevance of most of these latter substrates in GrB-mediated cell death has not yet been explored.

For GrH-induced cell death there is no general underlying mechanism known yet. To date, only two studies reported substrates and/or cell death pathways concerning this orphan granzyme, however, with contrasting results. Fellows et al. demonstrated that GrH induces cell death with typical hallmarks, such as loss of the mitochondrial membrane potential, ROS production, chromosomal condensation, and DNA degradation. Compared to GrB, GrH-mediated cell death is slower and does not involved cleavage of the pro-caspase-3, -7, -9, Bid, or ICAD, and neither release of cytochrome c [74]. In contrast, Hou et al. showed that cell death by GrH is induced by caspase-3 activation and cytochrome c release from the mitochondria. Moreover, ICAD and Bid were identified as direct GrH substrates [75]. Further studies are required to identify the physiological substrates and cell death routes for GrH.

GrK-induced cell death is mainly identical to the mechanism executed by GrA. Both granzymes display an almost similar P1-specificity, in that they cleave preferentially after a Lys or Arg [76]. Like GrA, GrK mediates ROS production in the mitochondria in a caspase-independent way and targets the same components of the SET-complex, leading to single strand DNA nicks [61,77]. Recently, a proteomic approach revealed that the macromolecular substrate-specificity of GrA and GrK overlaps only partially and, moreover, a substrate that is unique to GrK was identified, *i.e.* β-tubulin [78]. It has also been shown that GrK cleaves the known GrB-substrate Bid, which can induce caspase activation via cytochrome c release by mitochondria [79]. Whether these latter GrK-substrates are of physiological importance in GrK-induced cell death is yet unclear.

GrM is lately described as a granzyme that induces a distinct cell death pathway by a caspase-independent route. Several groups demonstrated that GrM-mediated cell death results in a typical morphology of target cells characterized by rapid cell swelling and subsequently lyses [80,81]. This autophagic-like phenotype is different compared to the other granzymes, indicating unique substrates and cell death pathways for GrM. Indeed, GrM-induced cell death does not feature obvious DNA fragmentation, occurs independently of caspases, and perturbation of mitochondria [80,81]. In contrast, the group of Fan reported that GrM promote cell death in a manner similar to GrB, including caspase activation, generation of ROS and cytochrome c release [82,83]. Whereas the latter group demonstrated direct cleavage of ICAD, PARP, and HSP75 (TRAP-1) by GrM, Cullen et al. identified nucleophosmin and PAK 2 as GrM-substrates [80,82,83]. As GrM is produced as a recombinant protein in different ways in the above mentioned studies, examination of cell death induced by native purified human GrM may determine the true mechanism behind the cell death mediated by this cytotoxic protease.

Besides the onset of target cell death by cleavage of intracellular substrates, granzymes also exert extracellular actions to induce cell death [84]. Although this has only been reported for GrA and GrB yet, it is likely that this is also the case for the other human granzymes. Granzymes may enter the extracellular compartment by either constitutive secretion, degranulation as a result of triggering by an inflammatory factor, and/or after misdirected release outside the immunological synapse during a cytotoxic response. Extracellular granzymes can mediate two major perforin-independent actions [84]. Depending on the local concentration of the granzyme it can then cleave extracellular matrix (ECM) proteins, such as fibronectin, vitronectin, and laminin, thereby inducing detachment of a target cell via disruption of integrin-mediated adhesion, finally resulting in anoikis [85,86]. In addition,

extracellular granzymes can cleave surface receptors that are important for cell survival and proliferation, thereby tipping the balance towards cell death [87]. Thus, human granzymes can induce cell death via various pathways, both by intracellular and extracellular mechanism. Other cell death-unrelated functions of granzymes and their physiological importance in diseases are summarized in another section.

Cellular expression of granzymes

Granzymes are differentially expressed by the various subsets of cytotoxic lymphocytes. Briefly, NK cells and activated $CD8^+$ T cells are the main cell types that express granzymes, whereas naïve T cells do not (Table 1). GrH and GrM are constitutively highly expressed in NK cells, NKT cells and γδ T cells, and only at low levels in cytotoxic CD8+ T cells, suggesting a role for these granzymes in the innate immune response [88-90]. In contrast, GrB is generally expressed in NK cells as well as activated $CD4^+$ and $CD8^+$ T cells, but also in regulatory and memory T cells [90-92]. GrA is mainly expressed by NK cells and $CD8^+$ T cells [91,92], whereas GrK demonstrates a preferential expression profile in lymphocyte subsets of the T-cell lineage [88,91]. Altogether, the granzyme expression studies harbor several discrepancies, which can partly be explained by the fact that different antibodies and techniques were used to detect the proteases: immunoblot analysis of highly purified sub-populations versus the more sensitive flow cytometry analysis. Nevertheless, the expression of human granzymes in the diverse populations of cytotoxic lymphocytes requires further investigation, including a more detailed characterization the granzyme expression patterns by effector and memory T cell subsets.

The differential protein expression of the human granzymes in the various cellular (sub) populations indicates a distinct regulation of these proteases. Interleukin 2 and probably other γ_c-dependent cytokines (*e.g.* IL-15 and IL-21) rapidly up-regulate GrA and GrB expression [93] Not much is known about the transcriptional regulation of the human granzymes, except some studies concerning GrB. Transcription factors that interact with the promoter region of the GrB gene and induce mRNA expression are Ikaros, CBF, NFAT, and NF-kB [94-96]. For the human GrA gene, only a functional glucocorticoid responsive element has been determined [97]. In addition to these transcription factors, post-transcriptional mechanisms are suggested to regulate the protein expression of granzymes, as cytotoxic lymphocytes usually express abundant levels of granzyme mRNA, allowing a rapid cytotoxic response upon stimulation [14]. In conclusion, the transcriptional regulation of the human granzymes requires further investigation.

In addition to cytotoxic lymphocytes, granzymes are also expressed by certain non-lymphoid cell types. For the human granzymes, this has only been demonstrated for GrB, although a broader expression pattern of especially the orphan granzymes has not been investigated yet [14,43]. Both GrB mRNA and protein are expressed in several human myeloid immune cells, *i.e.* plasmacytoid dendritic cells and basophils [98,99], whereas its expression in neutrophils is controversial [100,101]. In addition, GrB is also expressed in specific cells of the human reproductive system [102]. The specific expression in GrB in these different cell types, usually without detectable protein levels of perforin, suggests a non-cytotoxic and/or extracellular function for GrB in these conditions [84,103].

Table 1. Characteristics of the human granzymes.

	GrA	GrB	GrH	GrK	GrM
Synonyms	Gr1 CTLA3 HFSP	Gr2 CTLA1 CGL1	CGL2	Gr3 TRYP2	Met-ase 1
Chromosomal localization	5q11-q12	14q11.2	14q11.2	5q11-q12	19p13.3
Cleavage specificity	Arg >> Lys	Asp >> Glu	Phe >> Tyr	Lys >> Arg	Leu >> Met
Macromolecular substrates	SET Ape1 HMG2 Histone H1 Lamin A & C Ku70 NDUFS3	Pro-caspase-3,-7,-8,-10 Bid ICAD PARP α-Tubulin Rock II Lamin B DBP (adenovirus)	La ICAD (?) Bid (?) 100K (adenovirus) DBP (adenovirus)	SET Ape1 HMG2 Bid β-Tubulin	ICAD PARP (?) HSP75 Nucleophosmin SERPINB9
Cell death pathways	ROS-mediated Caspase-independent DNA-nicking	Cytochrome c release Caspase-dependent DNA fragmentation	Mitochondria-mediated DNA damage	ROS-mediated Caspase-independent DNA-nicking	Caspase-independent Autophagic-like
Cellular expression	NK cells NKT cells γδ T cells $CD8^+$ T cells $CD4^+$ T cells	NK cells NKT cells γδ T cells $CD8^+$ T cells $CD4^+$ T cells Regulatory T cells pDC Basophils	NK cells $CD4^+$ T cells $CD8^+$ T cells	NKT cells γδ T cells $CD8^+$ T cells $CD4^+$ T cells	NK cells NKT cells γδ T cells $CD8^+$ T cells
Endogenous intra-cellular inhibitor	Unknown	SERPINB9	Unknown	Unknown	Unknown

Granzymes in diseases

Together with perforin, granzymes can induce cell death of target cells, *e.g.* malignant cells and viral-infected cells. A non-functional granule-exocytosis pathway, as a result of mutations in the perforin gene or impaired granule secretion, underlies several clinical syndromes [104]. However, patients with a mutation in or deletion of a human granzyme gene are unknown so far. Moreover, mice deficient in one or even more of the granzymes revealed only minor difficulties in their capacity of tumor clearance, suggesting that granzymes are highly redundant and act as a back-up mechanism for each other, although this remains controversial [105-109]. In contrast, a role of granzymes in the clearance of pathogens is more generally accepted, as diverse granzyme knock out mouse models demonstrated an increased susceptibility to specific viruses [110-112]. Besides a probably impaired cytotoxic lymphocyte-induced cell death of viral-infected cell, these mice seems to combat increased levels of viral replication, because normally, most granzymes can cleave both viral and/or host proteins that are crucial in this process [103,113,114]. Recently, a low concentration of GrA was shown to promote rather an inflammatory response than inducing target cell death [57]. GrA induced the production of IL-1β and other pro-inflammatory cytokines in monocytes, suggesting that at least this granzyme is also involved in direct responses against pathogens by the innate immune system.

Interestingly, granzymes can be used as diagnostic tools, as their extracellular levels in plasma and other body fluids significantly increase in several inflammatory responses, *e.g.* rheumatoid arthritis, viral infection, transplant rejection, and shock [84,115,116]. Moreover, high expressions of granzymes associate with disease progression or even a poor clinical outcome of patients suffering from various types of lymphomas or inflammatory diseases [117-120]. Expression of GrA and GrB by inflamed tissues, *e.g.* acute transplant rejections, represents the amount of infiltrating cytotoxic lymphocytes [117,121]. So, granzymes are important proteases that usually play a host-protective role in diverse types of diseases.

Granzyme inhibitors

Direct inhibitors of granzymes can prevent unwanted proteolytic damage of these enzymes, although only few inhibitors are known for the human granzymes. The endogenous inhibitors can be divided into two groups based on their localization: intracellular and extracellular. So far, only one intracellular granzyme-inhibitor is identified, *i.e.* the serine protease inhibitor SERPINB9. SERPINB9 directly inhibits the proteolytic activity of human GrB, and moreover, cytoplasmic SERPINB9 protects cell from GrB-induced cell death [122]. Interestingly, SERPINB9 is a substrate for human GrM *in vitro*, which raises the possibility that GrM clears the way for GrB-induced cell death by inactivation of SERPINB9 [123]. Remarkably, endogenous intracellular inhibitors for the other four human granzymes are still unknown. In contrast to the single intracellular inhibitor, several extracellular granzyme-inhibitors have been identified. In plasma, GrA can be inhibited by α2-macroglobulin and SERPINC1 (anti-thrombin III) [124,125], whereas inter-alpha-trypsine inhibitor (ITI) and bikunin neutralize GrK activity [126]. GrM forms typical SDS-stable complexes with SERPINA1 (α1-antitrypsin) and SERPINA3 (α1-antichymotrypsin) *in vitro*, suggesting that these abundant plasma proteins might inhibit the activity of extracellular GrM [123]. The efficiency of inhibition of GrB by SERPINA1 is controversial [127,128].

In addition to the endogenous granzyme-inhibitors, several pathogens also express proteins that can inhibit members of this family of serine proteases and thereby escape

from a granzyme-mediated attack. CrmA, a cowpox-virus serpin, and adenovirus protein 100K can both inhibit GrB activity, whereas ecotin, a protease inhibitor expressed in the *E.coli*, can inhibit the activity of GrM [123,129,130]. Besides these endogenous and exogenous inhibitors, several synthetic compounds have been developed to inhibit human granzymes. These inhibitors harbor potential therapeutic applications in case of the need for immune suppression, although the specificity of such inhibitors is limited [131].

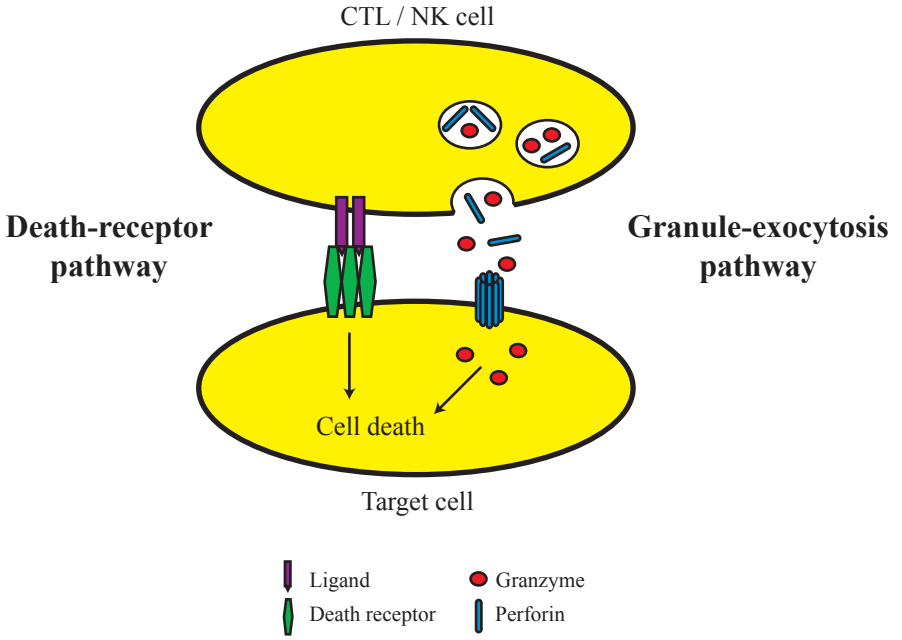

Figure 1. Cytotoxic lymphocytes can induce target cell death via two pathways. *Left: Death receptor pathway, in which ligand binding to a death receptor induces signaling routes resulting in cell death. Right: Granule-exocytosis pathway, in which perforin facilitates the entry of granzymes into a target cell. Intracellular, granzymes cleave specific substrates thereby inducing target cell death.*

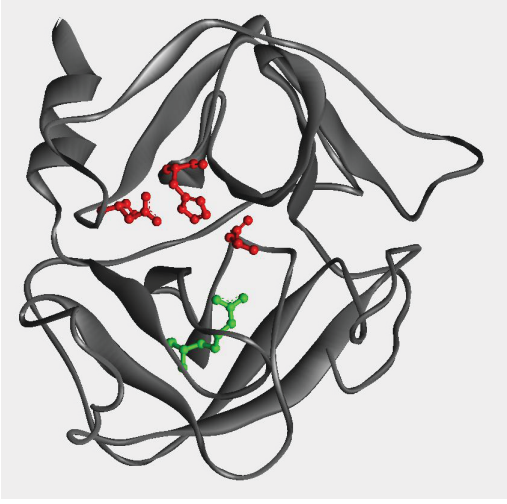

Figure 2. Crystal structure of human GrB. *The catalytic triad of His^{57}, Asp^{102}, and Ser^{195} is depicted in red. The GrB S1-residue, which largely determines cleavage specificity, is Arg^{226} (green). The substrate binding cleft is located in the horizontal middle of the granzyme. Figure modified from [37].*

SERPINS

Introduction

Many biological processes involve the enzymatic activity of proteases, *e.g.* blood coagulation, fibrinolysis, complement activation, digestion, extracellular matrix degradation, phagocytosis, activation of hormones, and apoptosis [132-134]. Tight regulation of the proteolytic activity in these processes is a critical requirement in the maintenance of homeostasis and to prevent excessive damage. First, most proteases are expressed as inactive pro-proteins. Their proteolytic function is usually initiated in a cascade-dependent manner, in which downstream pro-proteases are sequentially activated upon cleavage [135,136]. Second, active proteases can be controlled by specific protease inhibitors [137,138].

There are several super-families of protease inhibitors, corresponding to the various classes of proteases, of which the serine protease inhibitors (serpins) predominate. Serpins are widely dispersed and have been identified in plants, viruses, bacteria, prokaryotes, and eukaryotes [139]. The serpin family consists of over 500 members with SERPINA1 (α1-anti-trypsin), SERPINC1 (anti-thrombin III), and chicken ovalbumin as archetypes [140]. Currently, 36 human serpins are known that are further divided into 9 clades (A-I) based upon phylogenetic analyses [141,142]. The nomenclature of human serpins is as follow: serpin in capital letters, a capital letter representing the clade, and than a number, *e.g.* SERPINA1 [143]. Whereas most human serpins are located extracellular, serpins from the B-clade appears to be within the nucleo-cytoplasm. This thesis focuses primarily on these intracellular serpins.

Serpin structure and inhibitory mechanism

Serpins are characterized by a highly ordered tertiary structure despite an amino acid homology of only 30-50%. These proteins consist of about 350-400 amino acids, corresponding with molecular weights of 40-50 kDa, representing 3 β-sheets and 8 to 9 α-helices [144]. The unusual structure of this shared core domain determines the functional property of serpins, as they harbor a unique inhibitory mechanism. In contrast to the 'lock-and-key' / blocking type mechanism utilized by small protease inhibitors, serpins act as suicide pseudo-substrates for their target serine proteases. Upon cleavage by specific serine proteases serpins undergo a dramatic conformational change, finally resulting in a covalent irreversible complex of the two, in which the serine protease is inactive [145] (Figure 3).

One hallmark of serpins is their reactive center loop (RCL), a region of about 21 residues (P17-P4') that is localized on the outside of the tertiary core as an exposed helix [146]. In its native state, the RCL of the serpin is available to interact with a cognate serine protease. Moreover, the RCL contains amino acids (P1-P1') resembling the substrate sequence of a specific target serine protease [147]. So, the amino acid sequence in the RCL of a serpin largely determines which target serine protease can be inhibited. The high variety of these RCL-residues in-between serpins indicates that each serpin is specialized to inhibit one or more specific serine proteases (Table 2). In this way, different serpins together can inhibit a broad range of serine proteases. This is true even for the same serpin, as in some cases an alternative amino acid can be used as P1-residue in the RCL of a serpin [148,149]. This flexibility of the RCL broadens the inhibitory profile of a single serpin. In addition to the importance of the P1-residue, flanking amino acids (P4-P4') co-determine the specificity towards target serine proteases [150,151] and more distal residues (P15-P9; RCL hinge region) are also crucial for the inhibitory capacity of serpins [152].

In its native state, with the RCL folded outside of its core domain, the serpin resides in an energetically unfavorable conformation named the stressed form [153]. Upon docking of a cognate serine protease onto the RCL of a serpin and the formation of a Michaelis-like complex, the serine protease attacks the peptide bond between the P1 and P1'-residues [154,155] (Figure 3). In the normal situation of substrate-cleavage by a serine protease, the appearing covalent enzyme-substrate intermediate complex is further hydrolyzed, resulting in the release of the still active serine protease and leaving a cleaved substrate behind [46,156]. However, after cleavage of its RCL a serpin undergoes a rapid conformational change prior to hydrolysis of the ester linkage in the intermediate complex of the serine protease and the serpin [145]. This transition of the cleaved serpin, from the meta-stable stressed form into the hyper-stable relaxed form, is caused by its thermally labile structure and thus results in an energetically beneficial conformation. In detail, the amino-terminal part of the cleaved RCL inserts into the central β-sheet of the core domain of the serpin. Since the serine protease is still covalently linked to the RCL [157], the serine protease is moved to the opposite site of the serpin. Moreover, the stressed-to-relaxed transition of the serpin causes a distorted conformational structure of the serine protease, leaving it inactive and thus unable to still hydrolyze the covalent binding to the serpin [145]. Since the serpin has to be cleaved to fulfill its inhibitory function, the serpin is consumed as well in this process. So, finally the serine protease is trapped and forms a covalent complex with the serpin. Binding of the two proteins after complex formation is normally irreversible, based upon the highly favorable energetically conformation of the relaxed serpin [158]. Interestingly, these covalent complexes are SDS-stable and therefore serpin-serine protease interactions are rather easily to study using SDS-PAGE and immunoblot analyses.

The rate of insertion of the RCL determines the inhibitory efficiency of a serpin (Figure 4). If this rate is fast enough than the target serine protease will be trapped; however, when proteolysis of the serpin by the serine protease is completed before RCL insertion, then the still active protease is release, leaving a cleaved serpin behind. The efficiency of a serpin to inhibit its cognate serine protease is given by the stoichiometry of inhibition (SI) and apparent second order rate constant (k_{app}) [159]. The SI-value defines how many serpin-molecules are needed to inhibited one molecule of serine protease. Ideally, this ratio is 1.0 indicating that every single serpin traps one target serine protease. However, when a serpin is also a substrate in parallel to an inhibitor of its cognate serine protease, this is reflected by an SI-value greater than 1 [159]. The k_{app} defines the rate of inhibition when the association of the serine protease with the serpin is rate limiting and thus represents the continuous loss of serine protease activity in time. The reported k_{app}-values for serine protease-serpin interactions range from 10^2 to 10^8 M^{-1} s^{-1} [155]. The SI and k_{app} can be influenced by the tertiary structures of the proteins (*e.g.* RCL hinge region), temperature, pH, and ionic strength [155]. In addition, interaction with activating or bridging co-factors like the glycosaminoglycans (*e.g.* heparin), DNA, and extracellular matrix proteins can affect serpin-kinetics [160-162]. Serpin-serine protease interactions of biological significance usually have an SI-value near 1. In addition, serpins with high k_{app}–values are considered more effective, although local cellular concentrations eventually determine their physiological relevance.

Cellular expression of B-clade serpins

The human serpins that are expressed intracellular are represented by the B-clade sub-family. This subset of intracellular serpins was first proposed in 1993, based upon several

criteria including amino acid sequence identity, the presence of an interhelical CD-loop, and most significantly the lack of a cleavable N-terminal signal sequence [163]. Nowadays, the B-clade cohort consists of 13 members: SERPINB1-B13 [164] (Table 2). Their unique cellular distribution is both cytoplasmic as well as nuclear. Although B-clade serpins primarily reside intracellular and are short of a functional secretory sequence, several members (*e.g.* SERPINB2, -B3, B4, B8, and B9) are also detected extracellular, especially in the context of diseases [165-167]. For SERPINB3 and –B4, it is though that these serpins are not actively secreted, suggesting that they are passively released into the circulation, *e.g.* after necrosis of cells [168]. In contrast, secretion of SERPINB2 seems to be regulated because it can be induced by stimuli [169].

Initially, the cellular distribution of B-clade serpins was thought to be reflected by their chromosomal organization. In detail, the genes encoding for the 13 intracellular serpins map to two chromosomal clusters: 6p25 (SERPINB1, -B6, and –B9) and 18q21.3 (SERPINB2, -B3, -B4, -B5, -B7, -B8, -B10, -B11, -B12, and –B13) [164], as a results of several evolutionary inter- and intra-chromosomal duplications [170]. However, investigations of the serpin expression in various organs using PCR, northern blotting, or immunohistochemistry revealed a more complex transcriptional regulation. For example, immunohistochemical analysis of many different normal human tissues showed SERPINB8 expression by squamous epithelium,

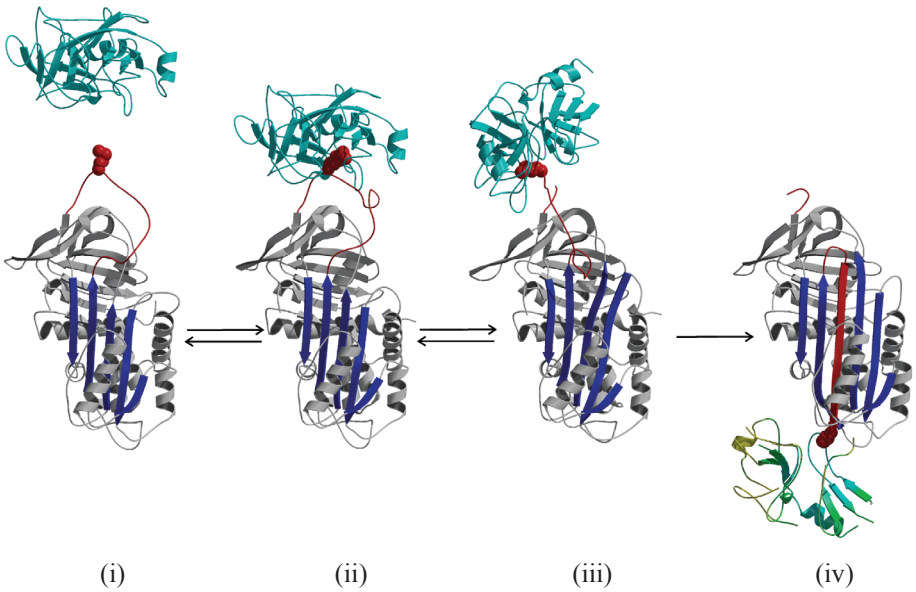

Figure 3. Structure of a serpin that undergoes a typical conformational change to form a complex with its target protease. *(i) Native structure of a protease (top) and a serpin (bottom; α1-antitrypsin) with its typical RCL including P1-residue (depicted in red). (ii) The protease encounters the RCL to form a reversible Michaelis-like complex. (iii) The protease cleaves the serpin in its RCL directly after the amino acid at the P1-position, starting the transition of the serpin structure. (iv) During the conformational change of the serpin, the RCL is inserted into the body of the serpin, resulting in a covalent interaction with the distorted and inactivated protease, of which the structure was detected for only 60%. Figure adapted from [17].*

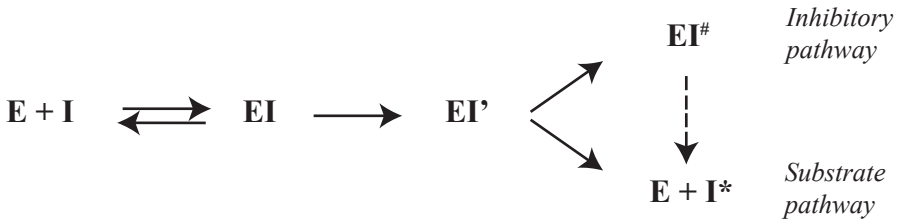

Figure 4. Branched pathways of the interaction of serpins with proteases. *E and I represent a protease (enzyme) and serpin (inhibitor), respectively. Initially, the two proteins form a reversible Michaelis-like complex (EI). Cleavage of the RCL by a protease results in an acyl-enzyme intermediate (EI'). Depending on the rate of RCL insertion by a serpin and hydrolysis by a protease, a serpin can either trap a protease in a covalent complex (EI#) or is a substrate, leaving behind a cleaved serpin and a free active protease (E + I*).*

monocytes, and neuroendocrine cells [171], whereas SERPINB9 was expressed by dendritic cells, cytotoxic lymphocytes, mast cells, and at immune privileged sites [172,173]. All together, intracellular serpins are expressed in a wide variety of tissues throughout the body. Moreover, almost each type of tissue expresses a different set of B-clade serpins [164]. Many serpins are expressed by the various epithelial layers. The three serpins clustered on chromosome 6p25 are, together with SERPINB2 and –B10, expressed by hematological and immunological cells [174,175]. Compared to normal non-neoplastic cells, expression of B-clade serpins is retained as well as down-regulated in transformed cells, depending on both the serpin and type of tumor.

Target proteases and functions of B-clade serpins

The main function of serpins is to inhibit the proteolytic activity of serine proteases, thereby influencing the outcome of many biological processes. *In vitro* inhibitory profile studies using purified proteins revealed many target serine protease-B-clade serpin combinations (Table 2). These combinations outnumber the amount of intracellular human serpins, as one single serpin can inhibit several serine proteases that display similar or even different specificities. In addition, some serpins are demonstrated *in vitro* to inhibit also proteases from another class, *i.e.* the cysteine proteases [176-178], a mechanism named cross-class inhibition. However, the physiological relevance of most of the potential target protease-serpin interactions has yet to be elucidated. Determination of specific serpin-mediated target protease inhibition *in vivo* has been complicated for two reasons. First, precise functional equivalents of human intracellular serpins often lack in model organisms such as the mouse, since the number of serpins is more diversified in the latter one [179,180]. Second, many cell types express several members of the intracellular serpins and these serpins appear to perform overlapping roles, resulting in redundancy in case one serpin is lacking or non-functional [164]. Nevertheless, the cellular distribution pattern of a B-clade serpin partly determines the corresponding target protease and thus its function. In general, a serpin is expressed in cells that also express its cognate target protease. In this way, serpins can counteract the aberrant activity of their endogenous target proteases. In addition, serpins can be protective against exogenous target proteases. These are the two main biological roles of serpins and will be further discussed.

Intracellular serpins are localized in the cytoplasm and the nucleus. In contrast, many target proteases are expressed in specific vesicular compartments, *e.g.* lysosomes or cytotoxic

Table 2. The human intracellular (B-clade) serpins. Table modified from [155,164].

Serpin	Synonyms	RCL P4-**P1**--P1'-P4'	(Potential) Target proteases
SERPINB1	PI2 Leukocyte elastase inhibitor (LEI) Monocyte/neutophil elastase inhibitor (MNEI)	ATF**C**--MLMP	neutrophil elastase, cathepsin G, proteinase 3, chymotrypsin, pancreatic elastase, and PSA
SERPINB2	Plasminogen activator inhibitor 2 (PAI-2) Placental PAI	MTG**R**--TGHG	uPa, tPA, acrosin, and plasmin
SERPINB3	Squamous cell carcinoma antigen 1 (SCCA-1)	GFG**S**--SPAS	cathepsin K, -L, -S, and -V
SERPINB4	Squamous cell carcinoma antigen 2 (SCCA-2) Leupin	VVE**L**--SSPS	cathepsin G and chymase
SERPINB5	PI5 Maspin	ILQ**H**--KDEL	?
SERPINB6	PI6 Cytoplasmic anti-protease 1 (CAP1) Placental thrombin inhibitor (PTI)	MMM**R**--CARF	thrombin, plasmin, trypsin, chymotrypsin, cathepsin G, uPA, factor Xa, and kallikrein 2
SERPINB7	Megsin	IVE**K**--QLPQ	plasmin
SERPINB8	PI8 Cytoplasmic anti-protease 2 (CAP2)	RNS**R**--CSRM	furin, chymotrypsin, subtilisin A, trypsin, factor Xa, and thrombin
SERPINB9	PI9 Cytoplasmic anti-protease 3 (CAP3)	VVA**E**--CCME	granzyme B, caspase 1, -4, -8, subtilisin A, and neutrophil elastase
SERPINB10	PI10 Bomapin	IDI**R**--IRVP	thrombin and trypsin
SERPINB11	Epipin	IAV**K**--SLPM	?
SERPINB12	Yokopin	VSE**R**--SLRS	trypsin and plasmin
SERPINB13	PI13 Hurpin Headpin	FTV**T**--SAPG	cathepsin K and -L

granules. It is generally though that in the cytoplasm B-clade serpins act to inhibit proteases that are leaking during their vesicular routing and/or degranulation [164]. Hereby, intracellular damage by aberrant activity of misdirected endogenous proteases is prevented. For example, SERPINB4 is mainly expressed by epithelial cells and inhibits the vesicular proteases cathepsin G and chymase [181,182]. In addition, SERPINB6 inhibits cathepsin G and β-tryptase in monocytes and mast cells, respectively [183,184]. SERPINB9 is expressed in the cytoplasm of cells containing the granule-associated enzyme granzyme B, like CTL, NK cells and mast cells [173,185]. SERPINB13 is expressed by epithelial cells of the oral mucosa and skin and is thought to inhibit the lysosomal proteases cathepsins K and L [186-188]. So, the main function of intracellular serpins is to regulate the proteolytic balance and protect cells against misdirected endogenous target proteases.

Besides protection against endogenous proteases, some B-clade serpins are capable to inhibit exogenous proteases that somehow cross the plasma membrane. This can be both human target proteases from other cells as well as proteases from pathogens. Antigen-presenting cells (APC), like maturated dendritic cells (DC), are usually in close proximity to and often have cell-to-cell contact with CTL. High expression of SERPINB9 by DC in their cytoplasm is an important mechanism to inactivate misdirected GrB from their neighbor CTL [189]. In this way, DC protect themselves against an exogenous apoptosis-inducing protease and remain capable to fulfill their function in such a cytotoxic environment. The same hypothesis holds true for SERPINB9-positive cells at diverse immune-privileged sites [172], as these tissues are otherwise highly vulnerable to inflammatory reactions. *In vitro* studies demonstrated that SERPINB8 and SERPINB9 also inhibit bacterial endoproteases, *e.g.* subtilisin A and furin [190,191]. SERPINB3 and SERIPNB4 are reported to respectively inhibit protozoa/helminth and house dust mite derived cysteine proteases [192,193]. If these serpins can protect cells against pathogenic infections has still to be elucidated.

Recently, evidence for alternative functions of B-clade serpins is emerging. Next to inhibition of target proteases in the cytoplasm, some intracellular serpins regulate processes at different locations, *i.e.* in the nucleus or even extracellular. Moreover, sometimes these actions are independent of their inhibitory function. The best studied example is SERPINB5, a non-inhibitory serpin that can inhibit angiogenesis [194]. Yeast two-hybrid analysis revealed that SERPINB5 interact with diverse proteins that are involved in the cellular stress response pathway, including several transcription factors [195]. SERPINB5 binds to other proteins via exosite-exosite interactions whereby its RCL sometimes regulate such interactions [196]. SERPINB2 mediates diversified biological functions, based upon its localization. Extracellular, SERPINB2 can inhibit urokinase-type plasminogen activator (uPA), thereby preventing the activation of plasmin and thus decreases cell migration and invasion [197]. In the nucleus, SERPINB2 can act as a retinoblastoma-binding protein, leading to reduced protein degradation of this tumor-suppressor gene and subsequently a decreased cell cycle progression [198]. In addition, several B-clade serpins can inhibit apoptosis, by interfering with various signal transduction proteins that normally lead to cell death, via non-inhibitory interactions or after translocation to the nucleus [199,200].

In conclusion, the function of intracellular serpins depends on their cellular expression and sub-localization but is mainly involved in protection against endogenous cognate proteases. However, the *in vivo* target proteases of quite some B-clade serpins remain to be established and these serpins may also fulfill protease-independent roles.

Serpins in diseases

When the delicate balance between the proteolytic activity of a protease and the expression of its cognate serpin is disturbed, this can lead to or strengthen pathological conditions. However, diseases caused by a non-functional B-clade serpin have never been reported, either because this would be lethal or it does not affect phenotype because of the overlapping roles between the serpins. On the other hand, over-expression or down-regulation of several B-clade serpins has been reported for diverse types of tumors. For example, expression of the tumor-suppressor gene SERPINB5 is lost in advanced tumors of diverse origin [201,202], whereas levels of SERPINB3/4 increase upon advanced stages of squamous cell carcinomas of various organs [203,204]. An advantage of the aberrant expression of several serpins in tumors is that they can be used as diagnostic identification markers. Moreover, serpin expression levels even correlate with disease progression and clinical outcome for specific types of tumors and can thus be used as predictive markers. For example, the granzyme B-inhibitor SERPINB9 is highly expressed in stage III and IV melanomas, and B- and T-cells of patients with non-Hodgin and Hodgin lymphoma [171,205]. In the first case, the presence of SERPINB9 expression in the tumors correlated with a poor clinical outcome of the patients, most probably because SERPINB9-positive tumor cells are less vulnerable for the granzyme B-containing cytotoxic cells of the immune system. For SERPINB13, several studies have shown that its RNA and protein expression is down-regulated by squamous cell carcinomas of the head and neck region [188,206,207]. However, the clinical relevance of this finding regarding tumor progression and patient follow-up has not been evaluated yet. Thus, several serpins are used as a diagnostic identification or prognostic marker for specific types of tumors, although their physiological function remains to be elucidated. As proteases and their inhibitors are often involved in tumor progression, more investigation is required for the application of other serpins as tumor-markers as well. In some diseases, serpins are identified as the pathogenic factor themselves. Hereby, serpins polymerize with each other because of repeating intermolecular protein associations by means of beta-sheet expansions, resulting in serpin-multimers that are extremely stable and rapid propagate [208]. This process is named Serpinopathies and underlies diseases as emphysema, early-onset dementia and liver cirrhosis [209]. So far, this phenomenon has not been reported for B-clade serpins.

Scope of the thesis

This thesis provides novel insights into the physiological and pathological role of both granzymes and serpins in the fields of immunology and oncology. Besides a global introduction about granzymes and serpins in chapter 1 and a more specific review on GrM in chapter 2, the outline of this thesis can be generally divided into three parts.

First, we have investigated the cellular expression of human GrB and GrM in more detail. GrB expression by the various lymphoid populations is well studied and, moreover, GrB is often used as a cell-specific marker for cytotoxic lymphocytes. However, GrB has recently also been detected in some myeloid cell types. Since the intracellular GrB-inhibitor SERPINB9 is expressed by human mast cells, we wondered if GrB is also expressed by these multifunctional immune cells (chapter 3). For human GrM, only two studies have been reported to date concerning its cellular protein expression pattern, with some controversial results. In chapter 4, we describe the generation of a novel specific monoclonal antibody against GrM that enabled us not only to detect the distribution of GrM in peripheral lymphocyte subsets in more detail, but also the regulation of GrM expression by various stimuli.

Second, two biochemical studies have been performed to identify novel macromolecular substrates as well as an intracellular inhibitor of human GrM. GrM induces a distinct cell death pathway, of which the molecular mechanisms remain poorly understood. A proteomics approach was used to reveal novel GrM substrates, which were subsequently validated in GrM-mediated cell death (chapter 5). Of all five human granzymes, only an endogenous intracellular inhibitor is known for GrB, *i.e.* SERPINB9. Because of the restricted substrate specificity of GrM and its potency to rapidly induce cell death, our aim was to identify an intracellular inhibitor of GrM. The interaction and corresponding kinetic parameters between GrM and an intracellular serpin, selected by a candidate-based approach, were analyzed (chapter 6).

Third, the expression of two intracellular serpins in diverse panels of tumors as well as their use as a diagnostic or even prognostic marker is demonstrated. The expression of SERPINB13 is known to be down-regulated by squamous cell carcinomas of the head and neck region (HNSCC). However, the clinical relevance of this down-regulation has not been studied yet. Therefore, we determined SERPINB13 expression in a panel of HNSCC using a novel mAb and associated this with histopathological and clinical parameters of the tumors and with patient survival (chapter 7). A previous study by our group, finding strong SERPINB8 expression by several neuroendocrine cell types, prompted us to investigate the expression of this serpin by the islets of Langerhans as well as by pancreatic islet cell tumors. Because of its strong expression by the latter one, the use of SERPINB8 as a diagnostic marker for these tumors has been evaluated (chapter 8).

Finally, the current findings as well as some future study perspectives are discussed and integrated in chapter 9.

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Chapter 2

The biology of granzyme M: a serine protease with unique features

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Abstract

The granule-exocytosis pathway is the major mechanism for cytotoxic lymphocytes to kill tumor cells and virus-infected cells. Cytotoxic granules contain the pore-forming protein perforin and a set of structurally homologues serine proteases called granzymes. Perforin facilitates the entry of granzymes into a target cell, allowing these proteases to initiate distinct cell death routes by cleaving specific intracellular substrates. The family of granzymes consists of multiple members, of which granzyme A and granzyme B have been studied most extensively. Since the cloning of the granzyme M cDNA in the early 90s, it has remained an 'orphan' granzyme for many years and only during the past few years the interest in this protease has increased. Granzyme M appears to be a potent inducer of tumor cell death with morphological hallmarks that are unique amongst all granzymes. In this review, we summarize the characteristics of granzyme M that are currently known, including its cellular expression, substrate specificity, physiological functions, and inhibitors.

The biology of granzyme M: a serine protease with unique features

Introduction

Cytotoxic T lymphocytes and natural killer (NK) cells, *i.e.* cytotoxic lymphocytes, are the major players in the cellular immune response against virus-infected and tumor cells. Upon recognition of a target cell, cytotoxic lymphocytes induce cell death via two mechanisms: the death receptor pathway and the more important granule-exocytosis pathway [1,2]. In the granule-exocytosis pathway, activated cytotoxic lymphocytes release the content of their pre-formed granules into the immunological synapse between the effector and target cell. These cytotoxic granules contain the key players that induce cell death in a target cell, *i.e.* the pore-forming protein perforin and a family of serine proteases known as granzymes (granule-associated enzymes) [3,4]. While perforin facilitates the entry of the granzymes into a target cell, granzymes cleave specific intracellular substrates to initiate cell death routes in a target cell [5,6].

The family of human granzymes consists of 5 members: GrA, GrB, GrH, GrK, and GrM. This non-sequential nomenclature of the human granzymes results from the more diversified family of murine granzymes: GrA-G and GrK-N [7]. Granzymes are homologous in their amino acid sequences for about 40%, display similar trypsin-like structural elements, and share the catalytic triad residues His, Asp, and Ser [8]. Using this catalytic triad, each granzyme cleaves a set of substrates that overlap only partially, thereby initiating at least three distinct specialized cell death routes in a target cell. Whereas GrA and GrB have been studied extensively in both human and mice, elucidation of the specific characteristics of the other (orphan) granzymes has started only recently [7,9].

This review summarizes and discusses the currently known insights of the cellular expression, substrates, inhibitors, and physiological role of GrM.

Gene and protein characteristics

GrM (also called Met-ase or Met-1) has first been identified in rat NK cells [10], rapidly followed by cloning of its human and murine orthologue in the early 90s [11-13]. The human and murine GrM gene both consist of 5 exons and map to the neutrophil elastase cluster on chromosome 19p13.3 and 10C, respectively [12,14]. This chromosomal localisation is distinct from the other granzyme family members. Depending on the species, the GrM gene encodes 257 to 264 amino acids, including a leader peptide and the conserved catalytic triad His, Asp, and Ser [10,12]. Alignment of human GrM with either the murine or rat orthologue revealed an amino acid consensus of 69 and 66 percent (Figure 1), respectively, whereas the identity with other granzymes is less than 45 percent. Like all granzymes, GrM is initially synthesized as a zymogen and finally converted into an active protease inside secretory granules, resulting in the for granzymes characteristic N-terminal Ile-Ile-Gly-Gly amino acid motive [15]. This mature GrM protein is highly basic with an iso-electric point above 10, has a predictive molecular mass of 27.4 kDa, and harbours two to three potential N-linked glycosylation-sites [16]. The crystal structure of GrM from any species has not been solved yet. For this review, we have generated a homology model of human GrM (Figure 2).

Cellular expression

Initial studies have determined that GrM mRNA expression is restricted to NK cells [10,11,13]. Northern blot analysis for human GrM reveals high mRNA expression by the NK leukaemia

cell lines Lopez NK and YT as well as purified $CD3^{-}CD56^{+}$ large granular NK cells, whereas none to very low levels of mRNA could be detected in diverse other leukaemia/lymphoma cell lines, purified $CD3^{+}CD56^{-}$ peripheral blood T cells, and purified $CD3^{-}CD56^{+}$ small high-density NK cells [13,17]. In addition, transfection of a reporter construct containing the 5'-flanking promoter region of the rat GrM gene into rat T-lymphoma, basophilic leukemia, and large granular NK cell lines only demonstrates transcriptional activity in the latter one [18]. Unlike GrA, GrB, and GrK, GrM mRNA levels are unaffected after stimulation with either IL-2, IL-12, IL-15, or interferon alpha for 6, 12, and 18 hours in the human NK-92 cell line, indicating that GrM transcription is differentially regulated as compared to these other human granzymes [19]. Surprisingly, GrM mRNA and a more abundantly expressed alternatively spliced GrM transcript have been identified in photoreceptor cells of the retina in the mouse [20].

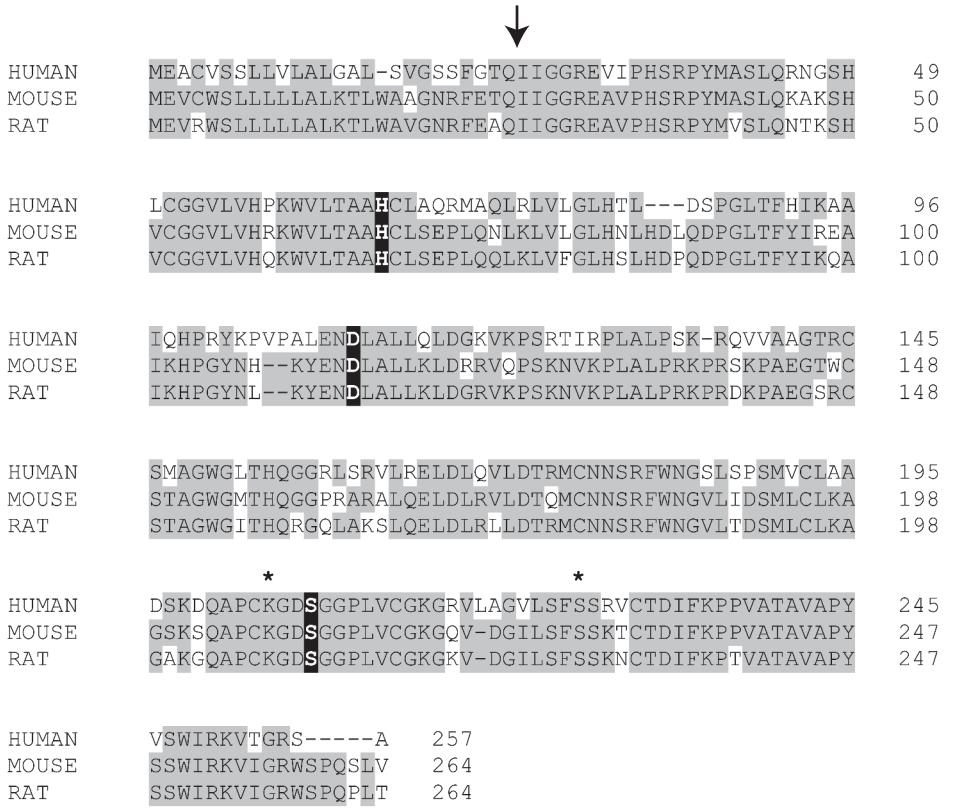

Figure 1. Alignment of human, mouse, and rat GrM. *Alignment of the amino acid sequence of full length human GrM with the sequences of mouse and rat GrM. Identity between the three sequences is represented in gray. The arrow points at the cleavage site in the GrM zymogen and the typical N-terminal motive Ile-Ile-Gly-Gly of mature granzymes. The conserved catalytic triad residues His^{66}, Asp^{111}, and Ser^{207} are indicated by a black background. The amino acids Lys^{204} and Ser^{226} that are located in the substrate-binding pocket of human GrM and restrict its cleavage specificity are depicted by *. The positions of the mentioned residues represent zymogen human GrM.*

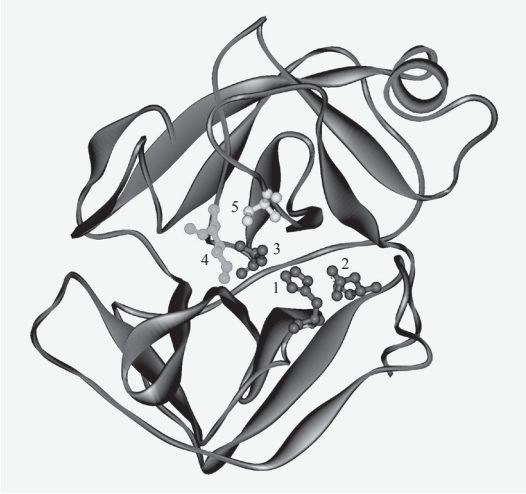

Figure 2. Homology model of human GrM. *A GrM homology model was generated on the Swiss Model Server [68], using human complement factor D as a template (PDB code 1DSTA). The catalytic triad residues His[66], Asp[111], and Ser[207] are indicated by number 1, 2, and 3, respectively. The amino acids Lys[204] (4) and Ser[226] (5) are located in close proximity to the Ser[207] residue in the substrate-binding pocket of human GrM and restrict its cleavage specificity.*

There are only two studies that have reported GrM expression by human lymphocyte subsets at the protein level. In analogy with the findings at mRNA level, Sayers *et al.* have demonstrated high GrM protein expression by purified $CD3^-CD56^+$ NK cells [21]. Protein expression is also detected in $CD3^+CD56^+$ NKT cells and γδ T cells, while it is absent in purified $CD4^+$ and $CD8^+$ T cells, suggesting a specific role for this granzyme in cells of the innate immune system [21]. Using flow cytometry analysis of peripheral blood mononuclear cells instead of western blotting and immunohistochemistry of sorted cell populations, Bade *et al.* have shown GrM protein expression by most NK cells (both $CD16^+CD56^+$ and $CD3^-CD56^{bright}$), NKT cells, and γδ T cells [22]. However, in contrast to Sayers *et al.* [21], GrM expression is also detected in about 35 percent of the $CD8^+$ T cells [22]. Because of this discrepancy, we have recently analysed human GrM expression in lymphocyte subsets by flow cytometry, using a novel GrM-specific monoclonal antibody, and real-time quantitative PCR. We have detected high expression of both GrM protein and mRNA in NK cells, NKT cells, γδ T cells, and $CD8^+$ T cells. GrM expression by $CD8^+$ T cells increased during differentiation from naïve into effector cells (de Koning *et al.* manuscript submitted). Together with Bade *et al.* [22], this finding challenges the restricted expression of human GrM to cells of the innate immune system and suggests a role for GrM in adaptive immune cells as well. Compared to other cytotoxic proteins, the expression pattern of human GrM in lymphocyte subsets overlaps mostly with GrA and perforin [21,22]. The human NK cell lines NK-92 and KHYG-1 both express high levels of GrM protein [21,23], which make these cell lines interesting tools to study GrM function, although it should be noted that these cell lines also express GrB [23]. Whether GrM, like GrB [24-26], is expressed by cell types of the myeloid lineage remains to be elucidated.

The cellular GrM protein expression is retained in various types of leukaemias, *i.e.* nasal NK/T cell, γδ T cell, and intestinal T cell lymphomas, as well as T cell and NK cell large granular lymphocytic leukaemias [27,28]. At that time, GrM expression suggested that all these types of tumors originate from innate immune cell types, which was unexpected for most of the T cell malignancies. However, the novel insights on GrM expression by CD8+ T cells request for re-interpretation of these two immunohistochemical studies. GrM expression in haematological malignancies seems to be similar as reported for GrB [29].

Substrate specificity

GrM was initially designated Met-1 or Met-ase, because of its unique substrate activity amongst serine proteases in NK cell granules, in that it cleaves synthetic peptides after P1-Met [10,30]. Peptide substrates containing other long narrow aliphatic hydrophobic amino acids, *i.e.* Leu and non-physiologic Nle, at this position are also hydrolysed by GrM of human, mouse, and rat origin [10,11,31]. A candidate-based approach of mutated amino acids in the catalytic centre of human GrM has defined that Lys^{204} and Ser^{226} (numbered residue Lys^{179} and Ser^{201} in mature human GrM) are of equal importance in determining the substrate specificity of GrM for at least P1-Met [31]. In our human GrM homology model, these residues are indeed positioned in the substrate-binding pocket in close proximity to the catalytic Ser residue (Figure 2). Advanced positional scanning of the GrM substrate specificity with a tetrapeptide library has revealed a far stronger preference for Leu over Met at P1 [32]. In addition, a strong specificity to Pro over Ala has been determined at the P2 position, whereas P3 displays a broad preference, *e.g.* Ala, Ser, Asp, Val, Glu, Phe, and Tyr, and partly depends on the P1 residue [32,33]. With a Lys as optimal amino acid at P4, the kinetically most favourable peptide substrate sequence for GrM has been identified as Lys-Val-Pro-Leu (P4-P1) [32]. Moreover, this sequence is highly specific for GrM as it is not hydrolysed by other homologues proteases, *i.e.* all four other human granzymes, neutrophil elastase, cathepsin G, and chymotrypsin [32,34]. In previous studies, when antibodies to GrM protein were not generated yet, this distinct cleavage specificity of GrM has been used to investigate which cellular subsets display Met-ase activity and thus likely express GrM protein. Hydrolysis of a P1-Met peptide by specific cell lysates indeed associates with GrM mRNA expression in both NK cell lines and sorted primary NK cells [13,17]. In contrast to other granzymes, Met-ase activity is lacking in haematopoietic progenitor cell-derived NK cells [35].

Induction of cell death

The capacity of GrM to rapidly promote cell death has been demonstrated recently, although some aspects of this process are still controversial. Several groups now have shown that loading of tumor cells with perforin or pore-forming analogues together with purified recombinant human GrM results in a unique cell death-morphology of the target cells, characterized by rapid cell swelling, formation of large cytoplasmic vacuoles, chromatin condensation with only slight segmentation of the nuclei, and finally lyses of the cells [36-38]. This necrosis/autophagy-like phenotype is different as compared to the morphology of dying cells upon attack by the other granzymes, pointing to the initiation of a unique cell death pathway by GrM, most likely via cleavage of distinct macromolecular substrates. Indeed, GrM-induced cell death does not feature obvious DNA fragmentation, and occurs independently of both caspase-activation and perturbation of mitochondria [36-38]. The pan-caspase inhibitor Z-VAD-fmk does not affect GrM-induced cell death and either caspase-3,

nor caspase-7, -8, and -9, are activated by GrM [36-38]. In addition, the cytoskeleton components α-tubulin and ezrin, the multifunctional nucleolar phosphoprotein nucleophosmin (NPM), and the apoptosis-related p21-activated kinase 2 (PAK-2) have been identified as direct and *in vivo* substrates of human GrM [36,37]. Site-directed mutagenesis and mass spectrometry analysis, respectively, have revealed that GrM hydrolyses α-tubulin and NPM both after a Leu-residue, which is in concordance with its cleavage-site specificity as determined by synthetic peptides [36,37]. Most of these substrates are not specifically cleaved by GrM only, since GrB also hydrolyses α-tubulin and NPM [39-41]. Nevertheless, GrM and GrB cleave these macromolecular substrates with different kinetics at different sites, resulting in distinct cleavage-products that may differ in their functionality [36,37]. These notions may underlie the unique morphological hallmarks of GrM-induced cell death.

In contrast to the necrosis/autophagic-like phenotype and corresponding macromolecular substrates of GrM-induced cell death, the group of Fan has reported that human GrM promotes cell death in a manner similar to GrB, including caspase-3 activation, DNA fragmentation, generation of reactive oxygen species, and cytochrome c release from the mitochondria [42,43]. In these studies, GrM directly degrades HSP-75 (TRAP1), inhibitor of caspase-activated DNase (ICAD), and poly(ADP-ribose) polymerase (PARP) [42,43], of which the latter two are also direct substrates of GrB and are directly reminiscent to DNA fragmentation [44,45]. Using N-terminal sequencing, the GrM-cleavage site in ICAD has been identified as Ser^{107} [43], which is in contrast to other reports regarding GrM-substrate specificity [30,32,36,37]. Since the studies concerning GrM-induced cell death and its macromolecular substrates make use of purified recombinant human GrM protein that is produced in different ways, is incubated at wide-spread concentrations, and in combination with various membrane-perturbing reagents to enter a target cell, further investigation is required to reconcile the true mechanism behind the cell death-mechanism initiated by this unique cytotoxic protease. In this context, it should be mentioned that our proteomic approach has recently revealed more potential GrM substrates, *e.g.* proteins involved in the cell stress response and the translation machinery, although these substrates remain to be verified *in vivo* [36].

Another interesting function of GrM may be to cleave and inactivate the physiological GrB-inhibitor serine protease inhibitor SERPINB9, as has been determined by *in vitro* analysis using purified proteins [32]. Although the physiological relevance of this finding remains to be established, it has been proposed that GrM can overcome SERPINB9-mediated inhibition of GrB and thereby clears the way for efficient GrB-induced target cell death. Whether GrM, like GrA, GrB, and GrK [46-49], is present in the circulation and hydrolyses extracellular substrates resulting in perforin-independent cell death or displays potential other functions also remains an intriguing question.

Inhibitors

The proteolytic activity and corresponding physiological function of a protease can be modulated by a specific protease inhibitor. Several studies have analyzed the inhibitory capacity of general (serine) protease inhibitors and specific synthetic peptides towards GrM, which have revealed the synthetic compound Z-Met^{P}(OPh-4-Cl)$_2$ and the bacterial-expressed serine protease inhibitor ecotin as the only known potent inhibitors of GrM activity [32,33,37,50,51]. The very poor inhibitory potency of all other tested compounds towards GrM might be caused by the restricted cleavage specificity of GrM towards Leu or Met at the P1-position and the rather small space at its active site [33]. For Z-Met^{P}(OPh-4-Cl)$_2$ it should be noted that

this peptide phosphonate also negatively affects the functionality of the pore-forming protein perforin [50], whereas ecotin is a broad spectrum serine protease inhibitor, thereby precluding their use as highly specific inhibitors of human GrM.

For GrM, only few natural endogenous inhibitors are known yet, which can be divided based upon their localisation, *i.e.* intra- or extracellular. A physiological intracellular inhibitor has so far only been described for murine GrM. The serine protease inhibitor (serpin) involved in cytotoxic inhibition (SPI-CI) forms a typical serpin-protease SDS-stable complex with murine GrM and has been proposed as an immune escape protein for GrM-induced cell death [52]. Although ectopic SPI-CI expression in the murine tumor cell line MFF is not sufficient to resist cytotoxic granule-induced cytolysis, it protects these cells from membranolysis induced by purified perforin in combination with murine GrM. In addition, knockdown of the endogenous expression of both SPI-CI and the murine GrB-inhibitor SPI-6 in the murine colon carcinoma cell line CMT-93 shows increased cytolysis by effector cells as well as purified cytotoxic granules [52]. There is no human orthologue of SPI-CI known yet, however, we have recently observed that the human intracellular serpin SERPINB4 binds to and inhibits the proteolytic activity of human GrM (De Koning *et al.* manuscript in preparation). In analogy with the human GrB-inhibitor SERPINB9 [53-55], it might be possible that SERPINB4 expression protects tumor cells and/or cytotoxic lymphocytes from GrM-induced cell death.

Two extracellular human serpins, SERPINA1 (α1-antitrypsin) and SERPINA3 (α1-antichymotrypsin), have also been identified to inhibit human GrM activity by formation of SDS-stable complexes [32]. However, the physiological relevance of these extracellular inhibitors remain to be established, especially because it is currently unknown if GrM is present in the circulation or other body fluids [46].

Table 1. Characteristics of GrM.

Cellular protein expression	NK cells [21, 22, A] NKT cells [21, 22, A] γδ T cells [21, 22, A] $CD8^+$ T cells [22, A]
Macromolecular substrates	ICAD [43] PARP [43] HSP-75 [42] α-Tubulin [36] Ezrin [36] Nucleophosmin [37] PAK-2 [37] SERPINB9 [32]
Inhibitors	SPI-CI [52] SERPINB4 [B] SERPINA1 [32] SERPINA3 [32] Ecotin [32] $Z\text{-}Met^P(OPh\text{-}4\text{-}Cl)_2$ [50]

[A] *De Koning et al. manuscript submitted.*
[B] *De Koning et al. manuscript in preparation.*

GrM knockout mice

Perforin knockout mice are severely immunodeficient and compromised in their ability to kill virus-infected and tumor cells [56,57]. In contrast, mice deficient for one of the eleven murine granzymes or mice that even lack both GrA and the GrB-F cluster are generally able to handle most infections and cancers, although they display a subtle impairment in the defence against some viruses as compared with wild-type mice [58-60]. GrM knockout mice have been generated a few years ago [61]. Like mice deficient for GrA and/or GrB, GrM knockout mice show normal development and homeostasis of the cytotoxic lymphocytes and efficient cytotoxicity of NK cells towards tumor cells [61]. This further points to a functional redundancy of the granzyme system in that one granzyme can take over the function of another. Strikingly, however, GrM knockout mice are particularly more susceptible to murine cytomegalovirus infection, but not mouse pox ectromelia infection, as compared with wild-type mice [61]. This phenotype could be the result of selective viral inhibition of one or more of the other murine granzymes or that GrM, like GrH [62,63], possesses cell death-independent direct anti-viral activity. Further research is required to address these possibilities. Whether in humans GrM also plays a role in the defence against viruses is currently unknown. In this respect it should be noted that striking evidence for differences in substrate specificity between human and murine orthologue granzymes is emerging recently [64-67], indicating that care should be taken by extrapolation of granzyme functions from mice to human, although such a species-dependent cleavage specificity has not been studied for GrM yet.

Conclusions

The granzyme-mediated granule-exocytosis pathway is the major mechanism for cytotoxic lymphocytes to induce target cell death. Granzymes trigger at least three distinct cell death pathways, of which the GrM pathway is beginning to emerge. This redundancy has likely evolved to provide protection against viruses and tumor cells with multiple strategies for evading cell death. Amongst the family of granzymes, GrM is a unique serine protease in that it displays highly specific substrate specificity and initiates tumor cell death with distinct morphological hallmarks. Several macromolecular GrM-substrates have been identified, although many of them require further investigation regarding their relevance in target cell death. Whether GrM is crucial for killing of specific target cells, displays non-apoptotic physiological functions, plays a pivotal role in coping with specific virus infections, is present and active in the extracellular compartment, and/or contributes to certain pathological conditions itself currently remain challenging open questions that deserve further study.

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Chapter 3

Human mast cells produce and release the cytotoxic lymphocyte associated protease granzyme B upon activation

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Abstract

Mast cells are widely distributed throughout the body and express effector functions in allergic reactions, inflammatory diseases, and host defense. Activation of mast cells results in exocytosis of preformed chemical mediators and leads to novel synthesis and secretion of lipid mediators and cytokines. Here, we show that human mast cells also express and release the cytotoxic lymphocyte-associated protease, granzyme B. Granzyme B was active and localized in cytoplasmic granules, morphologically resembling those present in cytotoxic lymphocytes. Expression and release of granzyme B by mast cell-lines HMC-1 and LAD 2 and by cord blood- and mature skin-derived human mast cells depended on the mode of activation of these cells. In mast cell lines and cord blood derived mast cells, granzyme B expression was mainly induced by non-physiological stimuli (A23187 / PMA, Compound 48/80) and substance P. In contrast, mature skin-derived mast cells only produced granzyme B upon IgE-dependent stimulation. We conclude that granzyme B is expressed and released by human mast cells upon physiologic stimulation. This suggests a role for granzyme B as a novel mediator in mast cell biology.

Abbreviations

GrB	Granzyme B
CL	Cytotoxic lymphocyte
CBdMC	Cord blood-derived mast cell

Introduction

Granzymes are serine proteases expressed by cytotoxic lymphocytes (CLs), such as NK cells and CTLs and localized together with the pore-forming protein perforin in specific "cytotoxic granules". CLs achieve granule-mediated target cell apoptosis through the combined actions of granzyme A (GrA) and/or granzyme B (GrB) and perforin (reviewed by [1]). Perforin is essential for cytolysis, whereas the granzymes induce rapid target cell DNA fragmentation and apoptosis [2,3]. In recent years, evidence is growing that GrB is not only involved in target cell death but also displays extracellular functions [4-7].

Granzymes share high structural homology with mast cell proteases. Mast cells are multifunctional immune cells involved in diverse processes, such as the host defense against helminthic parasites [8], the pathogenesis of allergy and asthma, cardiovascular disorders, inflammation and cancer [9,10]. Although their involvement in these processes is not fully understood, secretion of active mediators by mast cells seems to play a central role. Important among these are the serine proteases chymase and tryptase [11].

In mice, the structural relationship between mast cell chymases and granzymes is illustrated by the fact that 4 mast cell chymase genes reside on chromosome 14, linked to a gene complex that also encodes for granzymes [12]. Few reports have described GrB mRNA expression in mouse mast cells [13] and in murine mastocytoma cell lines [14]. Kataoka and coworkers showed in mice a strain-dependent and dissimilar regulation of GrB expression in mouse mast cells and CLs by the microphthalmia transcription factor (MITF) [13,15]. Very recently, Il-3-mediated GrB induction was reported in human basophils [16]. However, it still needs to be elucidated whether an equivalent GrB gene expression and regulation exists in human mast cells.

In the present study, we demonstrate that human mast cells indeed express active GrB *in vitro* and *in vivo*. GrB localized in specific granules resembling those of CLs and was secreted upon activation. These findings implicate GrB as a novel mediator produced and released by human mast cells.

Materials and methods

Cell culture

Lymphokine-activated killer (LAK) cells [17], YT-Indy [18], LAD 2 [19] (a kind gift from Dr. A. Kirshenbaum) and human mast cell line-1 [20] (HMC-1; a kind gift from Dr. J. Butterfield, Mayo Clinic, Rochester, MN) were cultured as described previously. Cord blood-derived human mast cells (CBdMC) were essentially cultured as described [21] with modifications. Briefly, citrate-treated fresh umbilical cord blood was obtained at the UMC Utrecht after informed consent. $1x10^4$ CD133+ cells were selected using a MACS CD133+ cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany and suspended in IMDM, supplemented with 1 % insulin-transferrin-selenium-G-supplement, 100 U/ml Penicillin, 100 μg/ml Streptomycin (all Invitrogen, Paisley, UK) and 0,1 % BSA (Sigma, St. Louis, USA). Cells were mixed with a 10-fold volume of serum-free methylcellulose medium (MethoCult™ SF^{BIT} H4236; Stemcell Technologies, Vancouver, Canada), supplemented with 100 ng/ml human recombinant Stem Cell Factor (hr-SCF), 5 ng/ml hr-IL-3 (both Peprotech, London, UK) and $5x10^3$ U/ml hr-IL-6 (Sanquin Reagents, Amsterdam, The Netherlands). After 6 weeks,

cells were retrieved from the methylcellulose and cultured up to twelve weeks in IMDM supplemented with 100 ng/ml hr-SCF, $5x10^3$ U/ml hr-IL-6 and 10 % FCS (Bodinco, Alkmaar, The Netherlands). The resulting population consisted of 90% mast cells as determined with immunocytochemistry for tryptase and CD117.

Skin-derived mast cells were derived from surgically skin samples as described (approved by the Human Studies IRB at Virginia Commonwealth University) [22]. The tissue was cut into 1-2 mm fragments and digested with type 2 collagenase (1.5 mg/ml), hyaluronidase (0.7 mg/ml), and type 1 DNase (0.3 mg/ml) in HBSS for 2 h at 37°C. After filtering through a #80 mesh sieve and resuspension in HBSS containing 1% FCS and 10 mM HEPES cells were layered over a Percoll cushion and centrifuged at 700 x g at room temperature for 20 min. Enriched cell fractions were resuspended at a concentration of 1×10^6 cells/ml in serum-free AIM-V medium (Life Technologies, Rockville, MD) containing 100 ng/ml of rhSCF (a gift from Amgen Inc., Thousand Oaks, CA) and split into separate wells every 4 to 5 days. Cultures of skin-derived mast cells were maintained for up to 3 months and consisted for approximately 100% mast cells as assessed cytochemically by metachromatic staining with toluidine blue, and by flow cytometry with anti-FcεRI mAb 22E7, a kind gift from Dr. J. Kochan (Hoffman-La Roche Inc., Nutley, NJ) [23].

Cell activation

HMC-1, LAD 2 and CBdMC were activated with either calcium ionophore A23187 (350 ng/ml) and PMA (30 ng/ml), Substance P (1 μM), Compound 48/80 (1 μg/ml) or C5a (1 μg/ml; all Sigma, St. Louis, USA) for 24 h, unless indicated otherwise. For activation via FcεRI, cells were first incubated with IgE-myeloma (1 ug/ml; Calbiochem, San Diego, USA) for 24 h. After washing, receptor cross-linking was induced with 7,5 ug/ml anti- FcεRI (KPL, Gaithersburg, MD, USA) for 24 h. Skin-derived mast cells ($0.5x10^5$ cells) were washed and activated for 24 h at 37°C with mAb 22E7 (1 μg/ml), C5a (Sigma, 2 μg/ml) or Substance P (Sigma, 2 μM) as described previously [24]. After stimulation for 24 h, cells and supernatant were collected separately for further analysis. Cell pellets were used for mRNA isolation (see below).

RNA isolation and real time RT-PCR

Total RNA was isolated using the peqGOLD TriFast isolation kit (PeQLAB, Erlangen, Germany), except for the RNA of the skin-derived mast cells, which was isolated using the RNeasy kit (Qiagen, Valencia, CA). Messenger RNA was transcribed into cDNA using $pd(N)_6$ random hexamers (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and a SuperScript II, RNase H-reverse transcriptase kit (Invitrogen, Breda, The Netherlands). Gene expression was measured in the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using the following primers: GrA (sense: 5'-AGGTGGAAGAGACTCGTGCAA-3'; antisense: 5'-GGTCTCCGCATTTATTTTCAAG-3'), GrB (sense: 5'-GTGCGGTGGCTTCCTGATA-3'; antisense: 5'-GGTCGGCTCCTGTTCTTTGAT-3') Perforin (sense: 5'-TGGAGTGCCGCTT CTACAGTT-3'; antisense: 5'-GTGGGTGCCGTAGTTGGAGAT-3'). Primers specific for human 18S ribosomal RNA (sense: 5'-CGGCTACCACATCCAAGGAA-3'; antisense: 5'-GCTGG AATTACCGCGGCT-3') were included for reference gene amplification. Relative quantitation of gene expression was determined using the comparative C_T method as suggested by the manufacturers. All results were normalized with respect to the 18S rRNA control, and

expressed relative to the expression levels in LAK cells.

GrB ELISA, activity assay and western blot

GrB *antigen* levels were measured with a GrB ELISA [25]. The lower limit of sensitivity for the assay is 10 pg/ml. GrB *proteolytic activity* was measured with an enzyme capture assay: microtiter plates were coated with anti-GrB mAb GB-11 [25] (2 µg/ml in 0.1 M sodium carbonate/bicarbonate buffer, pH 5.5) overnight at room temperature and washed with PBS / 0.02 % (v/v) Tween. Cell lysate was incubated for 2 h, after which the plates were washed. Plates were then incubated with GrB-specific chromogenic substrate Ac-Ile-Glu-Thr-Asp-pNA (Alexis Biochemicals, Lausen, Switzerland; 0.4 mM in 50 mM Tris, 100 mM NaCl, and 0.1 % Tween (v/v), pH 7.4). GrB activity was measured for 24 h at 37ºC at an absorbance of 405 nm on a Titer-Tek Multiscan (Labsystems, Helsinki, Finland).

Western blot analysis was performed as described previously [26]. GrB was detected using mAb GB-4 (1µg/ml) followed by HRP-conjugated rabbit-anti-mouse Ig. Bound antibodies were visualized with a chemiluminescence development reagent (ECL-system; Amersham) according to the manufacturer's instructions.

Perforin antigen ELISA

Microtiter plates were coated with anti-perforin mAb B-E48 (1 µg/ml). Perforin was detected with biotinylated mAb anti-perforin B-D48 (1 µg/ml, both perforin mAbs were a kind gift from Diaclone, Besançon, France). Binding sites were blocked with high performance ELISA buffer (HPE, Sanquin, Amsterdam, the Netherlands) containing 1 % (v/v) normal mouse serum followed by 30 min of incubation with 100µL/well streptavidin-polymerized horseradish peroxidase. Bound peroxidase was visualized using 100 ug/mL 3,30,5,50-tetramethylbenzidine and 0.003% v/v, H2O2 in 0.1 M sodiumacetate buffer, pH 5.5, as substrate. The reaction was stopped by 2 M H2SO4 to the wells and absorbance at 450 nm was read on a Titer-Tek Multiscan plate reader. All samples and standards (purified perforin; a kind gift from Dr. C.J. Froelich) were diluted in HPE.

Subcellular fractionation of HMC-1 cells

HMC-1 cells ($40x10^6$ cells) were washed, homogenized in 20 mM Tris / 250 mM sucrose (pH 7.4) using a syringe (25G x ½"), and centrifuged for 2 minutes at 2400*g*. The supernatant was applied to a preformed sucrose density 0.1 M step gradient (0.4 M - 2.0 M in 20 mM Tris-HCl, pH 7.4) and run overnight in an ultracentrifuge, using a SW40 rotor, at 100,000*g* at 4 ºC. Fractions (0.5 ml) were collected and tested for the presence of GrB and tryptase by ELISA. Fractions were assayed for β-hexosaminidase by release of *p*-nitrophenol from the substrate *p*-nitrophenyl N-acetyl-β-D-glucosaminide as described [27]. Absorbance values were read at 405 nm.

Immunohistochemistry and confocal microscopy

Sections (4 µm thick) of formalin-fixed, paraffin embedded normal human tissues as well as biopsies from patients with cutaneous or systemic mastocytosis were obtained from the tissue bank of the Department of Pathology, UMC Utrecht. Tissue samples from 3 patients with cutaneous mastocytosis (mastocytoma of skin) and from 3 patients with systemic mastocytosis (skin biopsy of the patients) were processed as described above. These patients complied with recent WHO classification [28], and are further described in table I.

Table 1. Expression of GrB and SERPINB9 in lesional mast cells in different mastocytosis subtypes.

Case / diagnosis	Patient characteristics			% positive tumor cells (intensity)		
	Place biopsy	Sex	Age (y)	Tryptase	Granzyme B	SERPINB9 [31]
1. Mastocytoma	Skin leg	F	0.5	75-100 (++)	75-100 (++)	75-100 (++)
2. Mastocytoma	Skin face	F	2	75-100 (++)	75-100 (++)	75-100 (++)
3. Mastocytoma	Skin arm	M	1	75-100 (+)	75-100 (+/-)	75-100 (++)
4. Systemic mastocytosis	Skin trunk	F	53	75-100 (++)	75-100 (++)	75-100 (++)
5. Systemic mastocytosis	Skin unknown	F	44	75-100 (++)	75-100 (++)	50-75 (++)
6. Systemic mastocytosis	Skin trunk	M	59	75-100 (++)	75-100 (++)	75-100 (++)

Immunohistochemical staining of tissue sections was essentially performed as previously described [26]. The following Abs were used: anti-GrA mAb GA-4 [29], anti-GrB mAb GB-7 [29], anti-tryptase mAb AA1, rabbit anti-CD117 pAb (both DAKO, Glostrup, Denmark) and anti-chymase mAb CC1 (Biomeda, Foster City, CA, USA). Negative control slides were stained with IgG of the appropriate subclass/species. Double staining was performed as follows: after antigen retrieval (15 minutes boiling in 0.01 M Na-citrate pH 6 in a microwave oven), sections were pre-incubated with normal goat serum. GrB was visualized in green by subsequent incubations with biotinylated mAb GB-7 (IgG2a), FITC-labeled streptavidin (DAKO), rabbit-anti-FITC (DAKO) and Alexa 488-labeled goat-anti-rabbit (Molecular Probes, Leiden, the Netherlands). Tryptase was visualized in red, using subsequent incubations with mAb AA1 and TRITC-labeled goat-anti mouse IgG1 (Southern Biotech, Birmingham, Ala, USA). As control, irrelevant, isotype-matched primary antibodies were used. Images were recorded using a Zeiss LSM-510 confocal laser scanning microscope (Weesp, the Netherlands).

Immuno-electron microscopy

HMC-1 cells and skin tissue were fixed in 2 % (v/v) paraformaldehyde in 0.1 M phosphate buffer for 2 h. The fixative was removed and free aldehydes quenched at room temperature with 50 mM glycine in PBS. Cells were embedded in 10 % (v/v) gelatin, and prepared for ultrathin cryosectioning and immunogold labeling [30]. Ultrathin cryosections were single immuno-labeled with 10 nm protein A gold particles. The following mAbs were used: anti-GrB (GB-7) and anti-tryptase (AA1) and secondary rabbit anti-mouse IgG (DAKO, Glostrup, Denmark). As control, irrelevant, isotype-matched primary antibodies were used.

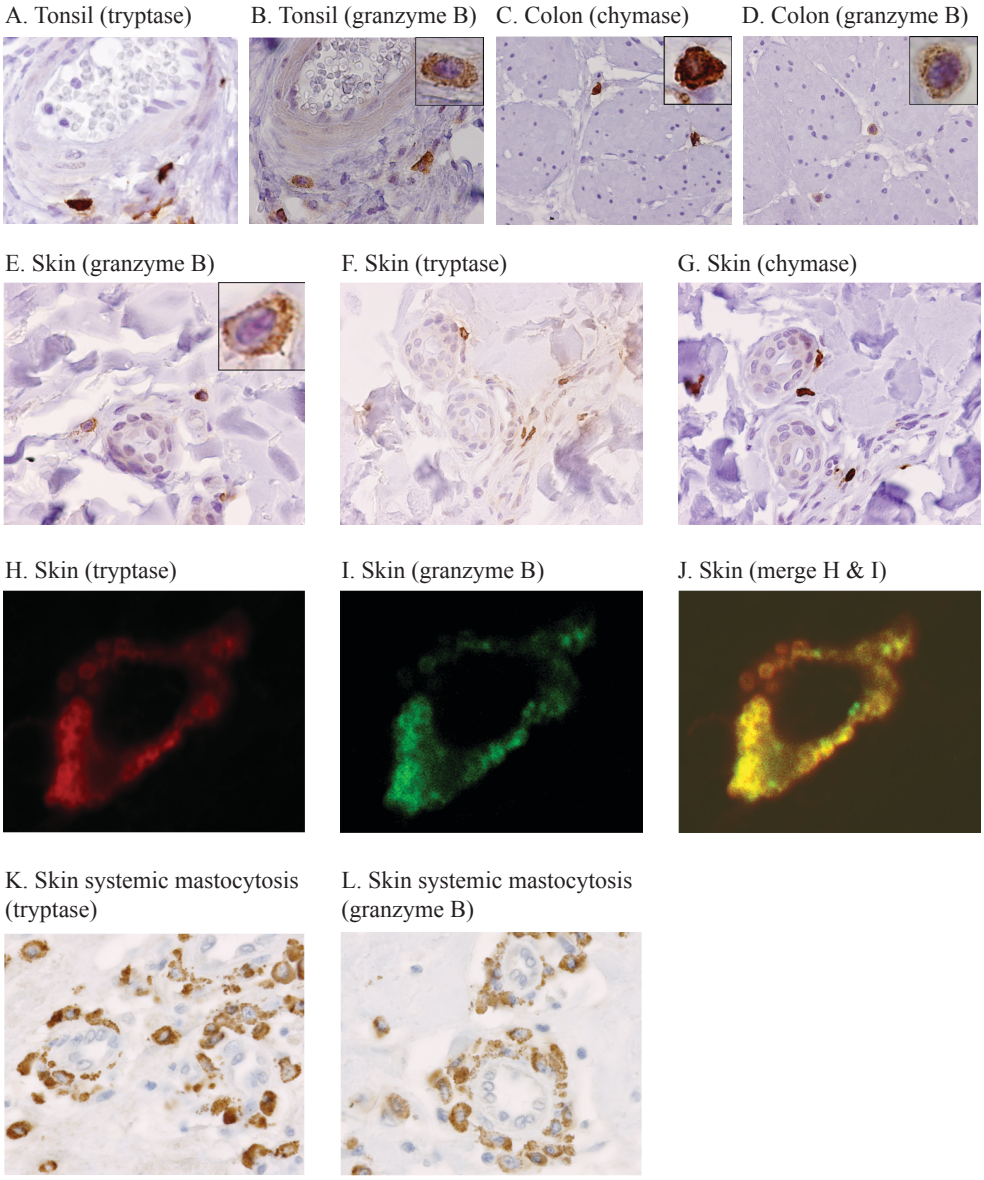
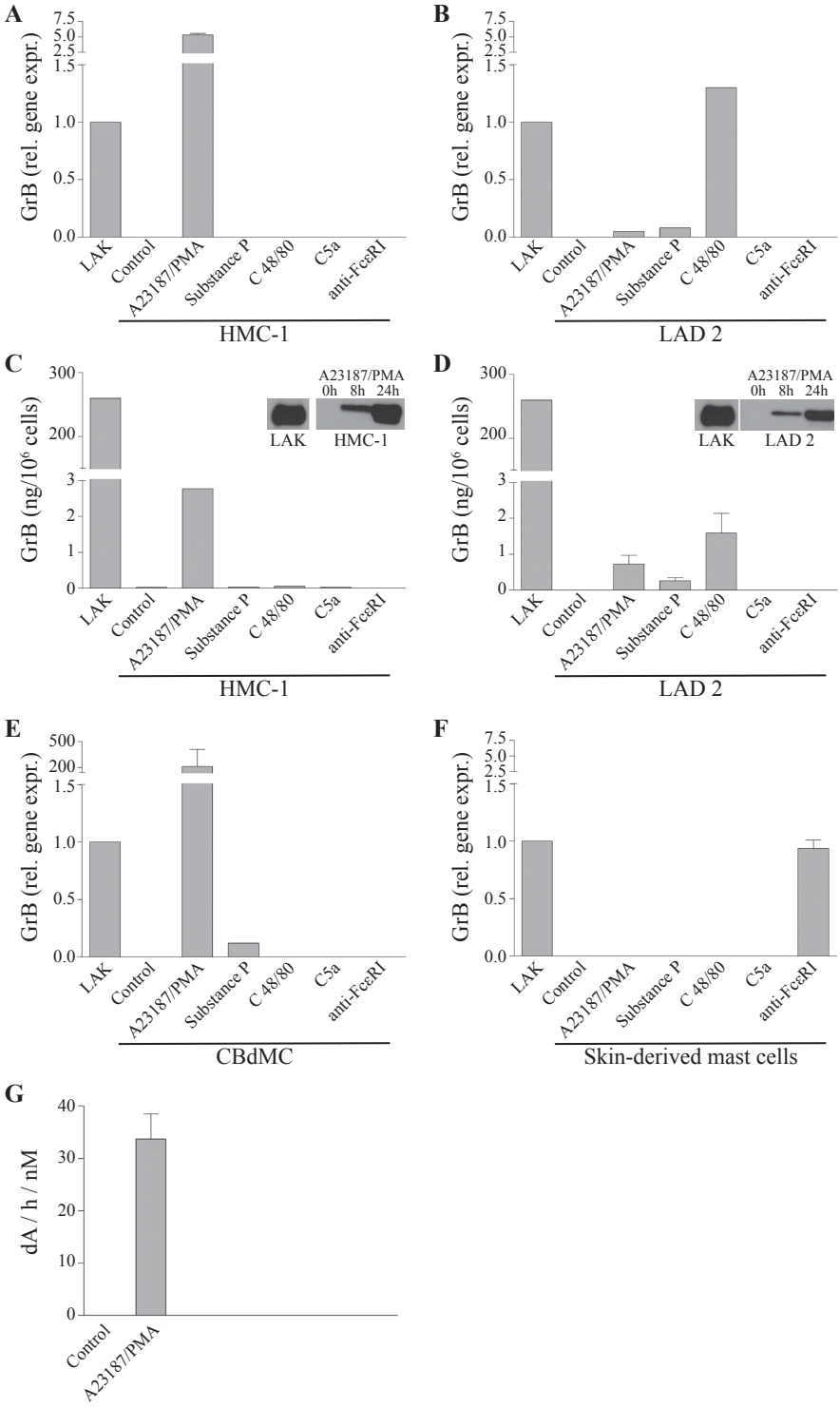


Figure 1. Human mast cells express GrB. *Tissue sections of tonsil (A, B), colon (C, D) and skin (E-J) were stained for chymase (mAb CC1: 1C, 1G), tryptase (mAb AA1: 1A, 1F, 1H), GrB (mAb GB-7: 1B, 1D, 1E, 1I) as described in M&M. Tissue sections of a systemic mastocytosis (case 9) were stained for tryptase (1K) and GrB (1L). Slides were stained with DAB and counterstained with hematoxylin. Magnification: 200 x (A-D), 400X (E-G, K, L). Figures 1H-J shows a confocal image of fluorescent double-staining of tryptase (Fig. H: red), GrB (Fig. 1I: green) in a skin mast cell (Fig. 1J: merged image). Magnification: 2500x.*


A
GrB (rel. gene expr.)
7.5
5.0
2.5
1.5
1.0
0.5
0.0
LAK
Control
A23187/PMA
Substance P
C 48/80
C5a
anti-FcεRI
HMC-1
B
GrB (rel. gene expr.)
LAK
Control
A23187/PMA
Substance P
C 48/80
C5a
anti-FcεRI
LAD 2
C
GrB (ng/10^6 cells)
300
200
3
2
1
0
A23187/PMA
0h 8h 24h
LAK
HMC-1
LAK
Control
A23187/PMA
Substance P
C 48/80
C5a
anti-FcεRI
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Control
A23187/PMA
Substance P
C 48/80
C5a
anti-FcεRI
LAD 2
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GrB (rel. gene expr.)
500
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1.5
1.0
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LAK
Control
A23187/PMA
Substance P
C 48/80
C5a
anti-FcεRI
CBdMC
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GrB (rel. gene expr.)
7.5
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LAK
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A23187/PMA
Substance P
C 48/80
C5a
anti-FcεRI
Skin-derived mast cells
G
dA / h / nM
40
30
20
10
0
Control
A23187/PMA

◄ ***Figure 2. Human mast cells express GrB upon stimulation.*** *Comparison of relative GrB mRNA levels of HMC-1 (A), LAD 2 (B), cord blood-derived (E; CBdMC) or skin-derived (F) mast cells either unstimulated or stimulated for 24 h with A23187 and PMA, substance P, compound 48/80, C5a or anti-FcεRI and LAK cells. Results are normalized to the internal control 18S rRNA and expressed relative to the value of LAK cells. (C, D) GrB protein levels in cell-lysates of above-mentioned HMC-1 (C) and LAD 2 (D) mast cell-lines as determined by ELISA. Values are presented as a representative experiment (n=3). Inset C/D: GrB Western blot of cells stimulated with A23187/PMA for 0, 8 or 24h. LAK cells were used as control. (G) Stimulated HMC-1 express proteolytically active GrB. GrB-specific activity is calculated from the rate of substrate (Ac-Ile-Glu-Thr-Asp-pNA) conversion (ΔA/h) and antigen concentration (nM) as measured by ELISA. (Data represent mean ± SEM of triplicate experiments).*

Results

Human mast cells express the cytotoxic lymphocyte-specific protein GrB

An immunohistochemical survey of normal human tissues showed GrB-positive cells that are much larger than CLs, in lymphoid tissue (tonsil: Figure 1A, B), the gastrointestinal tract (colon: Figure 1C, D), skin (Figure 1E-J), lung, subcutaneous tissue and other organs (not shown). These cells were identified as mast cells based on several observations. First, the GrB-positive cells showed a preferred tissue localization known for mast cells: around small vessels (Figure 1A, B), between smooth muscle cells (Figure 1C, D), and around skin appendages (Figure 1E-G). Second, GrB-positive cells morphologically resembled mast cells with their round, oval or fusiform appearance, a round central nucleus and abundant clearly demarcated cytoplasm (inset of Figure 1B, D, and E). Third, the identity of the GrB-positive cells was confirmed by sequential staining of sequential tissue slides with the mast cell specific markers tryptase (1A, F, H), chymase (1C, G) or CD117 (not shown). Finally, double staining of GrB with tryptase (Figure 1H: tryptase in red; 1I: GrB in green and 1J: merged) and CD117 (c-kit, not shown) was performed.

We also analyzed lesional mast cells of patients with cutaneous (n=3) or systemic mastocytosis (n=3) (Table I) for expression of GrB. In all cases the majority of lesional mast cells expressed abundant GrB. In general, GrB expression was much stronger compared to expression in normal mast cells. Figure 1 shows a representative example of tryptase and GrB expression in the skin biopsy of a patient with systemic mastocytosis (case 4, Figure 1K, L). The same biopsies were previously tested for the GrB inhibitor SERPINB9 and as expected co-expression of GrB and SERPINB9 is observed (Table I) [31]. Thus, not only normal human mast cells, but also neoplastic mast cells express GrB.

GrB antigen and activity is induced in human mast cell lines upon activation

The expression of GrB was further studied in the human mast cell lines, HMC-1 and LAD-2. Neither GrB mRNA nor GrB protein could be detected in the unstimulated HMC-1 and LAD-1 cells. However, HMC-1 mast cells expressed GrB mRNA and protein upon stimulation with A23187 and PMA. Treatment of HMC-1 with other mast cell stimuli (the neuropeptide substance P, compound 48/80, complement anaphylatoxin C5a or anti-FcεRI) did not induce GrB (Figure 2A and C). In contrast, in LAD 2 mast cells, GrB mRNA and protein was induced upon stimulation with A23187/PMA as well as with substance P or compound 48/80, whereas stimulation with C5a or anti-FcεRI had no effect (Figure 2B and D). After stimulation of HMC-1 and LAD 2 cells, GrB mRNA levels were even higher than those in LAK cells although the GrB protein levels were considerably lower. This may point

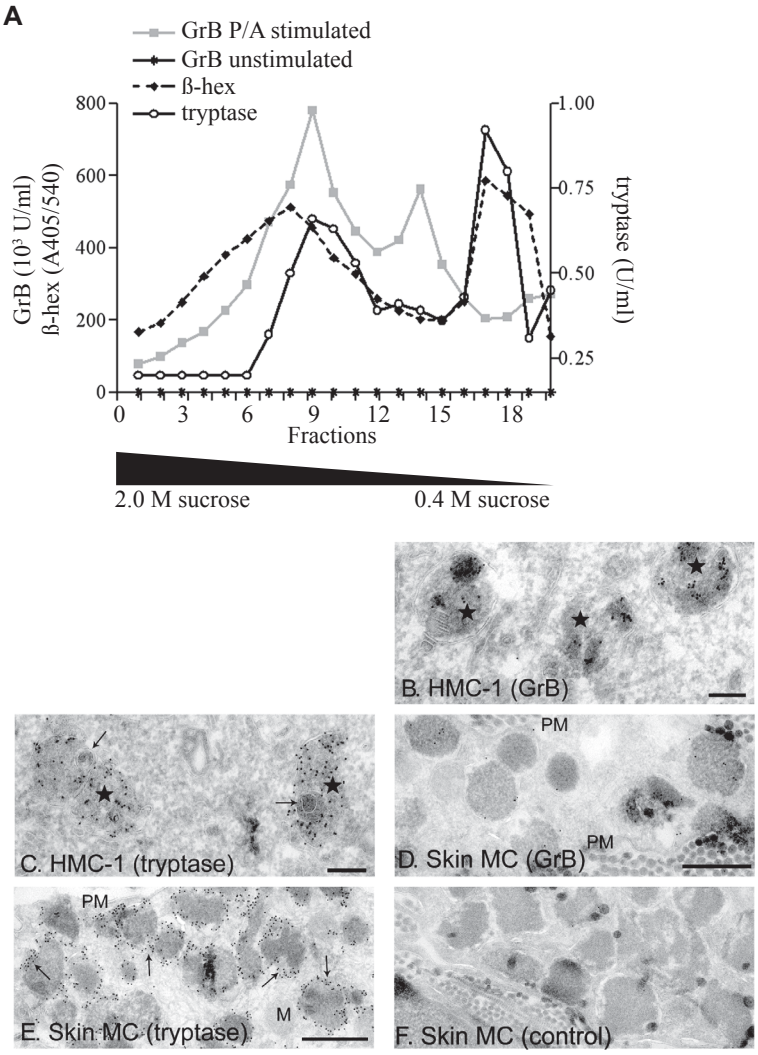

Figure 3. GrB is localized in mast cell granules that morphologically resemble cytotoxic granules in CLs. *(A) Sucrose density gradient analysis of HMC-1 mast cells. Cell homogenates were fractionated by a sucrose density step gradient. Density increases from right (0.4 M sucrose) to left (2.0 M sucrose). Fractions were analyzed for the presence of β-hexosaminidase activity (left axis, A405/540), GrB (left axis, 10^3 U/ml) or tryptase antigen (right axis, U/ml). Unstimulated HMC-1 mast cells were used for analysis of tryptase and β-hexosaminidase. HMC-1 mast cells stimulated for 24 h with A23187 and PMA were analyzed for GrB. HMC-1 mast cells (B,C) and 2 mm blocks of skin (D,E,F) were fixed and ultrathin cryosections immunolabeled for GrB (B,D) and tryptase (C,E) and a irrelevant, isotype-matched control antibody (F). (B) Electron micrograph of HMC-1 mast cell showing GrB labeling in several dense granules (stars). (C) Tryptase in HMC-1 mast cells is present in multivesicular structures (stars). Note that the dense area in these structures does not contain tryptase labeling (arrows). (D) GrB in human skin MC is present in the dense core of multiple granules. (E) Abundant tryptase labeling in dense granules in skin mast cell. The labeling is enriched in the space between the dense core and the enclosing membrane of the granule (arrows). (F) The negative control did not show granular labeling. PM, plasma membrane; M, mitochondrion; Bars, 200 nm.*

to a release by HMC-1 and LAD2 upon stimulation (see Figure 4) in contrast to the LAK cells which were not triggered to degranulate. Both LAD 2 and HMC-1 cells are derived from mast cell leukemia [19,32]. To confirm expression of GrB in non-malignant mast cells, we investigated mast cells cultured from either cord blood or skin tissue. Cord blood-derived mast cells showed induction of GrB mRNA upon stimulation with either A23187/PMA or substance P (Figure 2E), whereas GrB in skin-derived mast cells was induced upon stimulation with anti-FcεRI (Figure 2F). Because of the limited amount of mast cells, GrB antigen levels could not be determined. However, staining of cytospins preparations of A23187/PMA-activated LAD-2 and cord blood-derived mast cells showed a strong granular staining with the GrB antibody (results not shown). GrB expressed by activated HMC-1 cells is proteolytically active, as determined with an enzyme capture assay using the GrB-specific substrate Ac-Ile-Glu-Thr-Asp-pNA (Figure 2G).

Granzyme B and tryptase partially co-localize in mast cell granules with both lysosomal and secretory features

Immunohistochemical analysis of human mast cells showed a fine granular staining pattern of GrB in the mast cell cytoplasm (inset of Figure 1B, D, and E). This granular

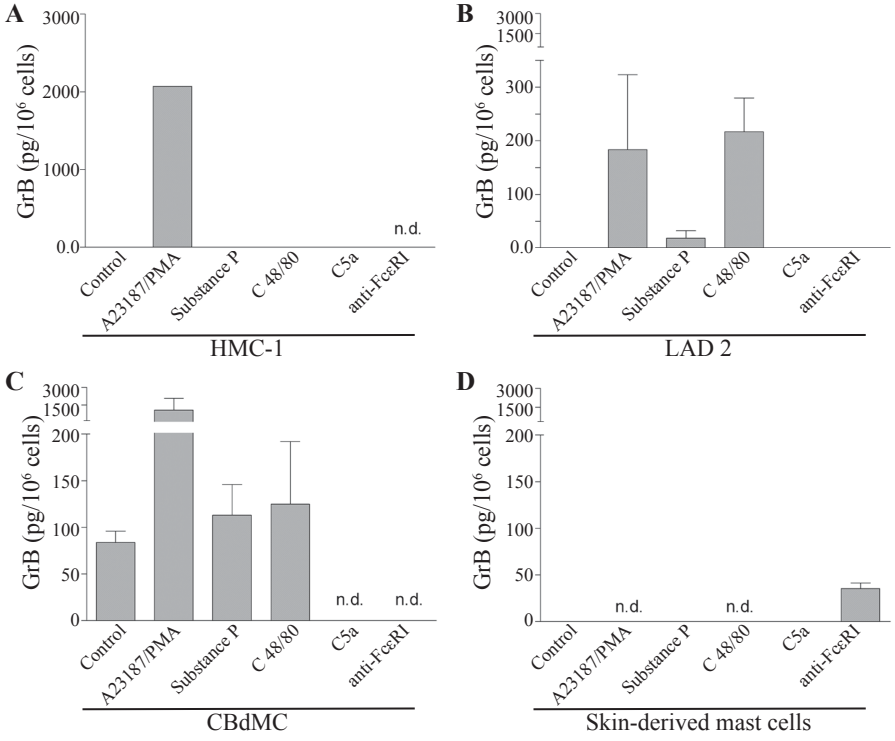

Figure 4. GrB is released upon mast cell activation. *(A, B) HMC-1 (A) and LAD 2 (B) mast cells were stimulated for 24 h with A23187 and PMA, substance P, compound 48/80, C5a or anti-FcεRI. Culture supernatants were analyzed for secreted GrB by ELISA. Values are presented as a representative experiment (n=3). (C, D) Cord-blood-derived mast cells (C) and skin-derived mast cells (D) were stimulated as described in Materials and methods. Culture supernatants were analyzed for secreted GrB by ELISA. Values are presented as a representative experiment (n=3).*

localization of GrB was confirmed by subcellular fractionation (Figure 3A) and immuno electron microscopy (Figure 3B-C) of stimulated HMC-1 mast cells. The major GrB peak co-migrated with tryptase and the lysosomal enzyme β-hexosaminidase, demonstrating GrB and tryptase localize in granules with similar density. These results are in line with the GrB and tryptase double-staining that showed extensive co-localization of these proteins in granules within tissue mast cells (Figure 1J). A smaller GrB peak was observed at lighter sucrose density, where only little tryptase or β-hexosaminidase were detected. Sucrose gradient fractions 15-18 represent the cytosol. These fractions also contain some granule-associated proteins probably due to their release from granules that were damaged during the homogenization procedure. As mentioned earlier, GrB could not be detected in unstimulated HMC-1 cells (-$_*$-).

Analysis at the ultrastructural level showed that both in HMC-1 cells and skin mast cells GrB accumulated in vacuolar structures with a dense content and few internal membranes (Figure 3B, D). Tryptase in HMC-1 mast cells was primarily enriched in vacuoles with internal membrane vesicles, resembling multivesicular late endosomes (Figure 3C). These structures, as well as the dense GrB-containing vacuoles, also labeled for the lysosomal membrane proteins CD63 and Lamp-1, and the lysosomal enzyme cathepsin D (data not shown). Irrelevant, isotype matched primary antibodies did not show granular staining (Figure 3F). Together with the sucrose gradient data, these findings suggest that both GrB- and tryptase-containing structures of stimulated HMC-1 cells are lysosomal granules, but that these proteins partially localize in granules with different morphology and density. In skin mast cells tryptase was found abundantly in structures with similar density as GrB. Interestingly, GrB appeared to be mainly located in the dense core, whereas tryptase was enriched in the space between the dense core and outer membrane of the granule (Figure 4E).

GrB is released upon mast cell triggering

Based on the granular localization of GrB in human mast cells we postulated that mast cells might release GrB upon proper stimulation. HMC-1 and LAD 2 mast cells indeed released their GrB content in the culture supernatant upon activation with the same stimuli that induced GrB mRNA and protein expression (Figure 4A and B, respectively). Accordingly, when a stimulus did not induce GrB expression, GrB could not be measured in the culture supernatant (C5a and anti-FcεRI). During stimulation, no increase in LDH activity in the culture supernatant was detected, excluding release of GrB by non-specific cell-lysis (results not shown). GrB was also released by cord blood-derived mast cells (CBdMC) upon stimulation with A23187/PMA and to a lesser extent upon stimulation with either compound 48/80 or substance P (Figure 4C). These results were comparable to the GrB release pattern of LAD 2 mast cells. In contrast to the mast cell lines and CBdMC, human mature skin-derived mast cells released GrB upon FcεRI-mediated stimulation but not upon stimulation with either substance P or C5a (Figure 4D). Because of the limited amount of isolated mast cells other stimuli were not tested.

HMC-1 but not other mast cells express low levels of perforin in vitro

To evaluate whether human mast cells also express other cytotoxic lymphocyte (CL)-specific proteins, HMC-1 and LAD 2 mast cells were analyzed for the expression of GrA and perforin. GrA, like GrB, is a granzyme expressed by human CLs and, together with GrB,

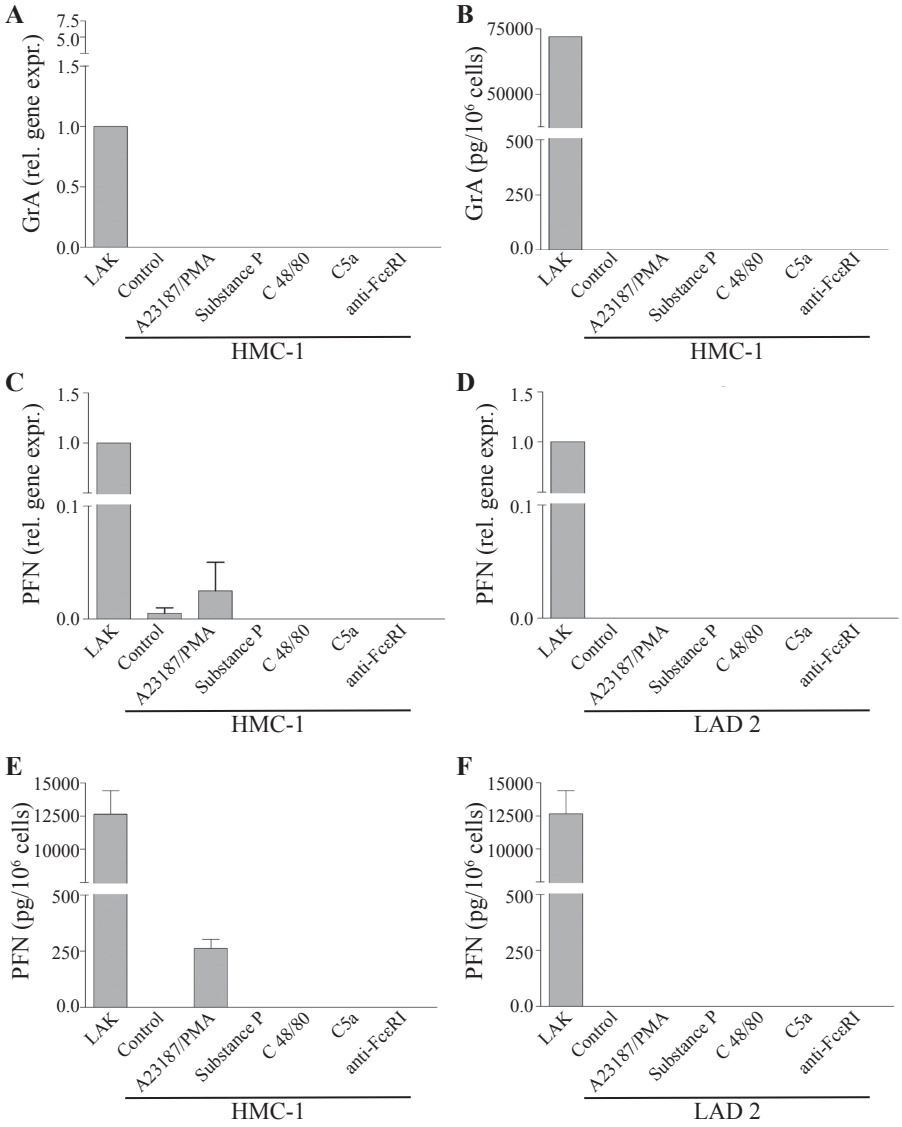

Figure 5. Perforin expression is restricted to the HMC-1 cell line. *(A) Comparison of relative GrA mRNA levels of HMC-1 mast cells either unstimulated or stimulated for 24 h with A23187 and PMA, substance P, compound 48/80 or C5a and LAK cells. Results are normalized to the internal control 18S rRNA and expressed relative to the value of LAK cells. (B) GrA protein levels in cell-lysates of above mentioned HMC-1 mast cell-lines as determined by ELISA. Values are presented as a representative experiment (n=3). (C, D) Relative perforin mRNA levels of HMC-1 (C) or LAD2 (D) mast cells either unstimulated or stimulated for 24 h with A23187 and PMA, substance P, compound 48/80, C5a or anti-FcεRI and LAK cells. Results are normalized to the internal control 18S rRNA and expressed relative to the value of LAK cells. (E, F) Perforin protein levels in cell-lysates of above mentioned HMC-1 (E) and LAD 2 (F) mast cells as determined by ELISA. Values are presented as a representative experiment (n=3).*

involved in CL-mediated cell death induction of target cells [1]. The pore forming protein perforin is required for cytolysis, which enables the granzymes to enter a target cell [3]. In contrast to GrB, no GrA mRNA or protein was detected in HMC-1 and LAD 2 mast cells, even upon stimulation, while LAK cells, as positive control, expressed both GrA mRNA and protein (Figure 5A and 5B, respectively). Very low levels of perforin mRNA (Figure 5C) and protein (Figure 5E) were detectable in HMC-1 mast cells only upon stimulation with A23187 and PMA. Perforin mRNA and protein could not be detected in either stimulated LAD 2 mast cells (Figure 5D and 5F, respectively) nor perforin mRNA in cord blood-derived or skin-derived mast cells (not shown). Although HMC-1 cells expressed perforin, these results indicate that perforin and GrA are not expressed by other (physiologic) mast cells, neither under normal conditions nor after (physiologic) stimulation.

Discussion

In this paper, we show for the first time expression of the protease GrB by both normal and neoplastic human mast cells *in vitro* and *in vivo*. Our results not only showed GrB expression in the 2 well characterized tumor-derived human mast cell-lines HMC-1 and LAD 2, but also in human cord blood-derived mast cells and mast cells isolated from the skin. In these cells, GrB mRNA, protein and proteolytic activity could be detected. GrB resided in granules and was released into the culture supernatant after stimulation with certain physiological and non-physiological stimuli. The relevance of these *in vitro* observations was supported by the constitutive GrB expression by mast cells present in normal human tissues. Also, neoplastic mast cells in biopsies from patients with mastocytoma showed high expression of GrB. These data may point to a novel function of human mast cells in host defense or in allergic disease.

For a long time, only NK cells and cytotoxic T-lymphocytes were considered to express GrB [1]. As such it is widely used as a marker to investigate the biological role of CLs in diseases such as transplant rejection and viral infection [25,33] and to identify specific subsets of lymphomas [34,35]. However, in recent years several publications reported GrB in other cell types as well. For example, GrB expression has been described in cells of the reproductive system, in developing spermatocytes and in placental trophoblasts [36], by granulosa cells of the human ovary [37] and in chondrocytes of articular cartilage [38]. Within the immune system, GrB expression was up-regulated in a subset of activated human dendritic cells [39]. Interestingly, expression of both GrB and perforin was recently reported in human neutrophils [40], but others could not reproduce this [41-43]. Very recently, IL-3 mediated GrB induction in human basophils has been reported [16]. Our finding that GrB is also expressed by human mast cells further challenges the specificity of GrB as marker for CL-mediated diseases.

Recently, we have shown that both normal and neoplastic mast cells express the GrB inhibitor SERPINB9 [31]. By scavenging GrB, SERPINB9 serves to protect CLs from death induced by their own, misdirected GrB [44]. SERPINB9 is also expressed at sites where degranulation of CTLs or NK cells is potentially deleterious and protects bystander cells from GrB released from neighbouring CLs during the immune response [45,46]. Like CLs, SERPINB9 is localized in the cytoplasm of HMC-1 and, similar to GrB, expression is induced after stimulation with A23187 and PMA [31]. In contrast, GrB was localized in granules as shown by immunohistochemical staining and subcellular fractionation. Interestingly, immuno-electron

microscopy showed that in mast cells GrB localized in granules morphologically resembling the cytotoxic granules of CLs. This strongly suggests that SERPINB9 serves to protect mast cells from their own misdirected endogenous GrB and hence enhance viability of the cells, similarly as observed for CLs [44,46].

The physiological function of GrB released from mast cells remains an intriguing question. In CLs, concomitant release of perforin is essential for GrB to induce apoptosis in the target cell (reviewed by [2,3]). Via perforin, GrB can access its substrates within the target cell cytoplasm and induce cell death. Only in A23187 and PMA stimulated HMC-1 low perforin mRNA and protein expression was observed. However, we did not find perforin mRNA transcripts and/or protein before or after stimulation in LAD 2, cord blood- or skin-derived mast cells. This is consistent with the observation that perforin expression could not be detected in mouse mast cells [47] or human basophils [16]. Thus, our findings indicate that mast cells in general do not possess the full cytolytic machinery necessary for the induction of apoptosis in target cells. In recent years, evidence is growing that GrB is not only involved in target cell death but can display extracellular functions as well. GrB has the ability to cleave and degrade extracellular substrates such as the cartilage proteoglycan, aggrecan [6], and is thought to contribute to joint destruction in rheumatoid arthritis [7]. GrB can also cleave the cell surface neuronal glutamate receptor, implicating it in the generation of auto-antigens [48]. Finally, GrB induces anoikis of primary and transformed human cells in the absence of perforin by cleaving extracellular proteins, such as fibronectin, vitronectin and laminin [4,5]. Thus, the GrB release from mast cells in the absence of perforin may indicate that GrB is involved in extracellular functions during mast cell activation.

Human mast cells can be activated by a large variety of stimuli, including allergens (via FcεRI), complement anaphylatoxins (C3a and C5a), neuropeptides (substance P) and lipoproteins (reviewed by [49]). These stimuli result in either release of a panel of pre-formed mediators, *de novo* generation of mediators, or a combination of both. The mast cell lines and the cord blood- and skin-derived mast cells tested all expressed and released GrB only upon stimulation. GrB expression was strongly increased upon A23187 and PMA stimulation. In addition, LAD 2 mast cells and cord-blood derived mast cells produced and released GrB upon stimulation with substance P while mature skin-derived mast cells showed GrB induction and release upon IgE-receptor cross-linking. Although C5a activates mast cells (data not shown) [50,51], C5a did not induce GrB synthesis and/or release. For the moment, we can only speculate on the (patho-) physiological conditions in which mast cell GrB may play a role. The neuropeptide substance P is an important mediator of the cross talk between nerves and mast cells in vivo. Bi-directional mast cell-nerve interactions are likely in mucosal tissues of many organs. Mediators produced by activated mast cells (e.g. histamine and TNF-α) can modulate neurotransmission [52] and, vice versa, neuropeptides (such as substance P) released from nerves can trigger degranulation of mast cells [53]. Next to its role in mast cell – nerve communication, substance P can be released from immune cells (such as T lymphocytes, monocytes, macrophages and eosinophils) [54,55]. In this view, mast cells could also be activated by immune cell-derived substance P to produce GrB. The observed constitutive GrB expression *in vivo* and the *in vitro* induction and release upon IgE-receptor stimulation may indicate a role for GrB as a novel mediator in mast cell-mediated allergic diseases. This putative role of GrB was recently highlighted by a publication of Tschopp *et al.* in which GrB induction was observed in isolated human basophils after IL-3 treatment [16]. GrB release was not only detected *in vitro* after IgE cross-linking, but also *in*

vivo in bronchiolar-alveolar lavage fluids from asthmatic patients after experimental allergen challenge. Future studies are necessary to delineate the contribution of mast cells to the GrB found at sites of allergic diseases.

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Chapter 4

The cytotoxic protease granzyme M is expressed by lymphocytes of both the innate and adaptive immune system

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Abstract

The cytotoxic serine protease granzyme M (GrM) is one of the five human granzymes, which are mainly expressed by cytotoxic T lymphocytes and/or NK cells. Upon perforin-dependent entry into a target cell, GrM cleaves specific substrates resulting in the onset of a unique cell death mechanism. However, the role of GrM in pathophysiological conditions is not clear yet. Knowledge of the expression and regulation of GrM by lymphocyte populations is instrumental for a better understanding of the contribution of this unique granzyme in health and disease. Two previous studies demonstrated GrM protein expression by lymphocytes of the innate immune system, i.e. NK cells, NKT cells, and γδ T cells, whereas its expression by $CD8^+$ T cells remained controversial. In the present study, we have investigated the expression and regulation of GrM in lymphocyte subsets in more detail. Flow cytometry analysis with a novel specific antibody against human GrM confirmed high expression of this protease by NK cells, NKT cells, and γδ T cells. $CD8^+$ T cells also expressed GrM and comparing the naive to early effector-memory, to late effector-memory, to effector subset, this expression gradually increased during differentiation. In contrast, $CD4^+$ T cells hardly expressed GrM. Quantitative PCR analysis for GrM mRNA levels in the diverse lymphocyte sub-populations confirmed the FACS results. GrM protein expression by lymphocyte populations was not significantly affected by a panel of GrB-inducing cytokines, indicating that GrM expression is differentially regulated as compared to GrB. In conclusion, the human cytotoxic protease GrM is, besides by innate immune cells, also expressed by $CD8^+$ effector T cells, in particular by the differentiated effector $CD27^-$ $CD45RO^-$ subset. Our current findings support not only a role for GrM in the innate but also in the adaptive immune response.

Abbreviations

CTL	Cytotoxic T-lymphocyte
Gr	Granzyme
mAb	Monoclonal antibody
NK cell	Natural killer cell
PBMC	Peripheral blood-derived mononuclear cells
PFN	Perforin
rh-IL	Recombinant human interleukin
RQ	Relative quantification

Introduction

Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells (i.e. cytotoxic lymphocytes) play a pivotal role in the effector arm of the immune response that eliminates virus-infected cells and tumor cells. Cytotoxic lymphocyte-induced killing is mediated by two major pathways: ligation of cell death receptors on targets cells and directed release of granules into the synapses between effector and target cells [1,2]. Cytotoxic granules primarily contain perforin (PFN) and a sub-family of serine proteases known as granzymes [3]. While PFN facilitates the entry of granzymes into the target cell, the latter induce cell death by cleaving critical intracellular substrates [4].

In humans, five granzymes (i.e. GrA, GrB, GrH, GrK, and GrM) are known and all induce cell death [5]. Whereas GrA and GrB have been studied extensively, the elucidation of the characteristics of the three orphan granzymes took off only recently [6]. The five human granzymes are homologous in their amino acid sequences for about 40%, therefore similar in structure, and share the catalytic triad residues His, Asp, and Ser [7]. On the other hand, these granzymes display differences in their substrates specificity, have distinct chromosomal localizations, and are differentially expressed by the various lymphocyte sub-populations [5]. The various granzymes can initiate different cell death pathways, of which the mechanism and corresponding morphology of GrM-induced cell death is unique amongst these cytotoxic proteases [8].

GrA and GrB are mainly expressed by $CD4^+$ and $CD8^+$ CTL and NK cells [9-11], although expression of GrB has also been reported for some myeloid cell types [12-14]. GrK is preferentially expressed by lymphocytes of the T cell lineage [9,15], whereas GrH expression is more restricted to NK cells [11]. For human GrM, only two studies have reported its cellular protein expression pattern yet. First, GrM expression has been shown to be restricted to NK cells, NKT cells, and γδ T cells, as determined by Western blotting of highly purified FACS-sorted leukocyte subsets [16]. These results were confirmed at single-cell level by Bade *et al.*, using flow cytometry [15]. However, the latter study also detected GrM expression by $CD8^+$ T cells, whereas Sayers *et al.* demonstrated that this sub-population lacked GrM expression [15,16]. To our knowledge, the regulation of GrM expression has not been investigated at the protein level yet.

Elucidation of the exact cellular expression pattern of human GrM and its regulation is of importance to understand which subsets can mediate GrM-induced cell death. The present discrepancy in literature, concerning the presence of human GrM in CTLs, prompted us to study the cellular GrM expression in more detail. We analysed the expression of GrM in different lymphocyte populations as well as its regulation by several stimuli in peripheral blood lymphocytes. For these purposes we generated a novel specific monoclonal antibody (mAb) against human GrM and detected expression of this protease by flow cytometry.

Material and methods

Recombinant granzyme proteins

Active recombinant human GrB, GrK, and GrM were expressed in *Pichia pastoris* and purified by cation-exchange chromatography as previously described [17,18]. When indicated, biotin-7-NHS (Roche, Mannheim, Germany) was conjugated to the recombinant human

granzymes, according to the manufacturers' instructions and dialysed to PBS.

Generation and selection of a specific mAb against human GrM

Immunization of mice, hybridoma culture, and selection of mAbs against GrM were performed as previously described with slight modifications [19,20]. Mice were successively immunized (Hybridoma facility, Faculty of Veterinary Sciences, Utrecht University, The Netherlands) with purified recombinant human GrM. The obtained hybridomas (n=386) were screened for affinity to soluble recombinant human GrM using an antibody-capture ELISA. Herein, Maxisorp 96 well microtiter plates (Nunc, Roskilde, Denmark) were coated with 5 µg/ml anti-mouse Ig mAb (clone 226; kindly provided by Dr. L.A. Aarden, Sanquin Research, Amsterdam, The Netherlands) to capture anti-GrM mAbs from the hybridoma supernatants. Biotin-conjugated recombinant human GrM (0.5 µg/ml) and HRP-conjugated streptavidin (Perkin-Elmer, Boston, MA) were subsequently added to detect mAbs with affinity for the soluble protease. In between, wells were washed throughout with PBS, 0.1% Tween-20. Presence of GrM was determined using TMB (Pierce, Rockford, IL) as a substrate according to the manufacturers' instructions and the absorption was measured at 450 nm using a microtiter plate reader (Anthos, Cambridge, UK). Positive clones (n=11) were screened in a second round by antibody-capture ELISA to preclude cross-reactivity with soluble recombinant human GrB and GrK, followed by limiting dilution. Finally, one clone, 4B2G4, was selected based on its high affinity against and specificity for recombinant and native human GrM as determined by the antibody-capture ELISA and flow cytometry. This mAb is of the IgG1-isotype and used throughout this study. Biotin-7-NHS (Roche) and AlexaFluor488 (Molecular Probes, Eugene, OR) were conjugated to the novel mAb, according to the manufacturers' instructions.

Cell culture, cell sorting, and stimulation

HeLa and Jurkat cells were maintained in DMEM and RPMI-1640 medium (Invitrogen, Paisley, UK), respectively, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma, St. Louis, MO), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). The human NK cell line KHYG-1 [21] was purchased from the Health Science Research Resources Bank (JCRB0156) of the Japan Health Sciences Foundation and cultured similar as Jurkat cells, with the addition of 50 ng/ml recombinant human interleukin 2 (rh-IL-2) (Wako, Osaka, Japan). Cell lysates were prepared by three freezing-thawing cycles in liquid nitrogen of cells resuspended in tris-buffered saline (20 mM Tris, pH 7.4, 150 mM NaCl). Protein concentrations of the supernatants were measured using the procedure of Bradford (Bio-Rad, Hercules, CA).

Peripheral blood-derived mononuclear cells (PBMC) from healthy adult blood donors were separated by Ficoll (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation. For cell sorting of specific T cell subsets, PBMC were first stained with a mixture of anti-CD3-PerCP (BD Biosciences, San Jose, CA), anti-CD8-PE (Sanquin, Amsterdam, The Netherlands), anti-CD27-APC (BD Biosciences), and anti-CD45RO-FITC (Invitrogen), whereas a combination of anti-CD3-PerCP and anti-CD56-PE (Sanquin) antibodies was used for NK cells. The indicated T-cell subsets and NK cells ($CD3^{-}CD56^{+}$) were sorted on a FACS Aria cell sorting system (BD Biosciences).

For stimulation experiments, PBMC were maintained for 3 and 6 days in complete RPMI medium ($1x10^{6}$ cells/ml), supplemented with 25 U/ml recombinant human interleuking (rh-

IL) 2 (Sanquin), 10 ng/ml rh-IL-6 (ImmunoTools), 10 ng/ml rh-IL-7 (Sigma), 4 ng/ml rh-IL-12 (ImmunoTools), 10 ng/ml rh-IL-15 (ImmunoTools), 50 ng/ml rh-IL-21 (PeproTech), 50 ng/ml rh-IL-27 (R&D Systems), 5 ng/ml interferon-α (PeproTech), or 10 ng/ml LPS (Sigma). Non-stimulated PBMC were included as a control and all ten conditions were also tested in combination with 1 μg/ml anti-CD3 (clone 1XE; Sanquin). Harvested cells were analysed by flow cytometry analysis for GrM expression by all indicated lymphocyte subsets.

Flow cytometry analysis

To detect GrM protein expression in diverse lymphocyte populations, PBMC were incubated with either one of the three (A-C) combinations of antibodies, as indicated in Table 1. Cells were first stained for membrane markers for 20 min at 4°C, washed with PBS, 0.5% BSA, and subsequently fixated and permeabilized with Cytofix/Cytoperm (BD Biosciences) for 10 min at 4°C. Next, two sequential intracellular stains were performed for 20 min at 4°C. In-between these incubations, cells were washed with Perm/Wash buffer (BD Biosciences). Irrelevant mouse IgG mAbs with appropriate conjugates (BD Biosciences) were used as isotype controls. Finally, cells were analyzed on a BD FACSCanto II, using FACSDiva software (BD Biosciences).

Table 1. Intracellular FACS staining. *Stimulated PBMC were analysed by flow cytometry according one out of three (A-C) combinations of three sequential incubations (cell membrane, intracellular I, and intracellular II) with labelled antibodies to detect GrM expression by either $CD4^+$ and $CD8^+$ T cells (A), specific T cell subsets (B), or NK cells (C). For GrM expression analysis our novel AlexaFluor488-conjugated mAb 4B2G4 was used. When indicated, GrB and PFN were also included. In PBMC stimulated with anti-CD3, the CD3-PerCP antibody was replaced by anti-CD4-PerCP (BD).*

	Cell membrane	**Intracellular I**	**Intracellular II**
T cells (A)	CD3-PerCP (BD Biosciences) CD8-APC (eBiosciences)	PFN-Biotin (Ancell)	Streptavidin-APC-Cy7 (BD) GrM-AF488 (clone 4B2G4) GrB-PE (Sanquin)
T cell subsets (B)	CD3-PerCP CD8-APC-AF750 (eBiosc.) CD27-APC (BioLegend) CD45RO-PE-Cy7 (BD) γδ-PE (BD)	-	GrM-AF488
NK cells (C)	CD3-PerCP CD56-PE (BD)	PFN-Biotin	Streptavidin-APC-Cy7 GrM-AF488

Real-time quantitative PCR (RT Q-PCR)

Total RNA was extracted from the sorted cell populations and KHYG-1 cells using the RNeasy Mini Kit (Qiagen, Hamburg, Germany), according to the manufacturer's instructions. Messenger RNA was transcribed into cDNA using oligo$(dT)_{15}$ (Promega, Phoenix, AZ) and random (Promega) primers, and SuperScript III Reverse Transcriptase (Invitrogen). For RT Q-PCR analysis, inventoried primer/probe combinations for human GrM (Hs00193417_m1), GrB (Hs01554355_m1), and PFN (Hs00169473_m1) were obtained from Applied Biosystems (Foster City, CA). Five μL of cDNA was mixed with 12.5 μL Taqman Universal Master Mix,

1.25 µL primer, and 6.25 µL Milli-Q. The PCR reactions were performed by the ABI-Prism 7900HT RT PCR System (Applied Biosystems). Thermal cycling constituted a denaturation step at 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 60 sec. All experiments were performed in duplicate and differences in the mean threshold cycle (Ct) values did not exceed 0.5. Data was analyzed using the relative quantification (RQ) ($2^{-\Delta\Delta Ct}$) method in the SDS 2.2.1 software (Applied Biosystems), in which $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{calibrator}$ and $\Delta Ct = Ct_{target} - Ct_{reference}$. As reference we used the GAPDH gene (Hs99999905_m1). A mRNA batch extracted from the KHYG-1 cell line was used as calibrator sample for normalization.

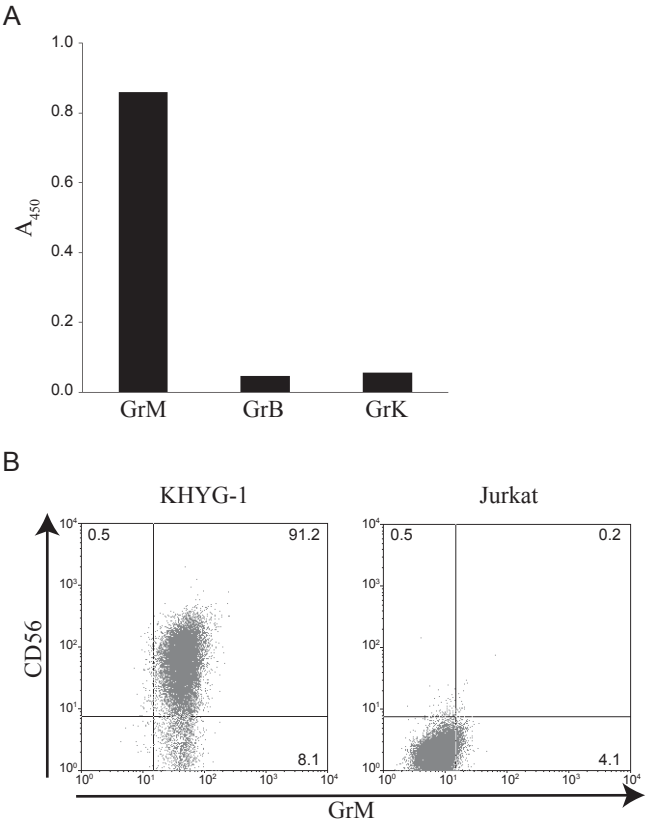


Figure 1. Characterization of a novel mAb against human GrM. *A) Affinity of mAb 4B2G4 for biotin-conjugated soluble recombinant human GrM, GrB, and GrK (0.5 µg/ml), as determined by an antibody-capture ELISA, in which anti-GrM mAb 4B2G4 was first captured by a coated anti-mouse Ig Ab. The absorptions (at 450 nm) of this ELISA are depicted for each granzyme and represent one out of three experiments with similar results. B) Flow cytometry analysis of intracellular GrM expression by KHYG-1 and Jurkat cells. Cells were labelled with anti-CD56-PE and anti-GrM-AF488 (clone 4B2G4) mAbs. Percentages of cells are indicated in each quadrant.*

Results

Characterization of a novel mAb against human GrM

To study the protein expression pattern of human GrM, we generated a novel mAb against this cytotoxic protease. Clone 4B2G4 was selected because this mAb demonstrated high affinity for soluble recombinant human GrM in an antibody-capture ELISA and showed no cross-reactivity towards soluble recombinant human GrB (49% amino acid homology) and GrK (52% homologous) (Figure 1A). Moreover, flow cytometry analysis, using AlexaFluor488-conjugated mAb 4B2G4 revealed a strong intracellular staining for native human GrM in the NK cell line KHYG-1 (Figure 1B, left panel). Almost all KHYG-1 cells expressed GrM. Appropriate-labelled isotype control mAbs and absence of staining of the GrM mRNA-negative Jurkat T cell line (Figure 1B, right panel) showed the specificity of this intracellular staining. CD56 was used as a cell membrane marker for NK cells and, in the case of KHYG-1 cells, 91.2% of the cells were double-positive for GrM and CD56 (Figure 1B, left panel). Taken together, mAb 4B2G4 demonstrated a high and specific affinity towards both soluble recombinant as well as native human GrM, and was therefore selected to detect GrM expression in lymphocyte subsets by flow cytometry.

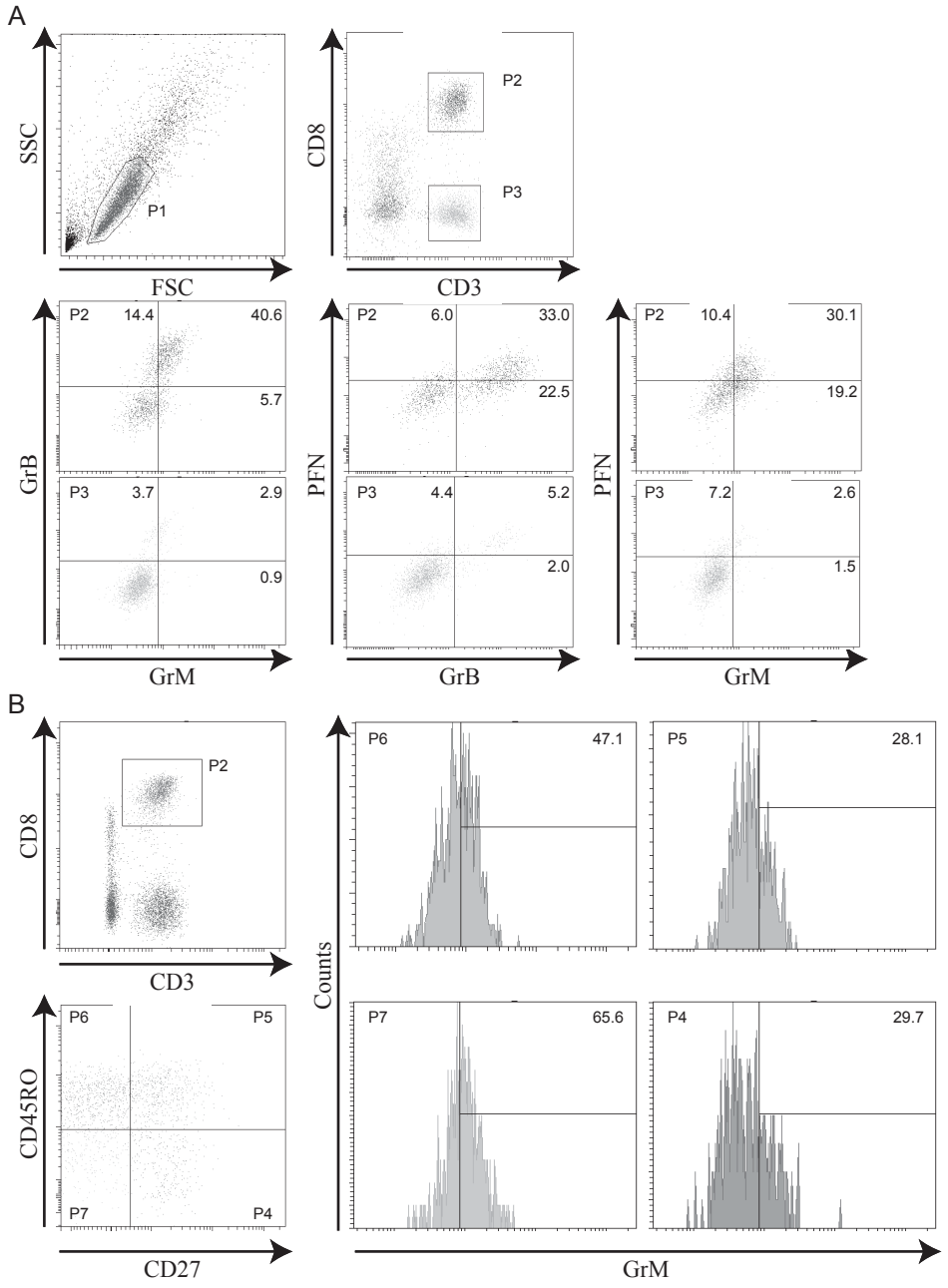
Protein expression of GrM, GrB, and PFN by lymphocytes

In line with the two previous studies [15,16], flow cytometry analysis on PBMC from healthy donors (n=4) showed that human GrM is highly expressed by NK cells ($CD3^-$ $CD56^+$), NKT cells ($CD3^+$ $CD56^+$), and γδ T cells ($CD3^-$ $\gamma\delta^+$) (Table 2). In all three cell types, GrM was expressed by 70 to 80 percent of the cells, of which γδ T cells displayed the highest intensity in GrM expression. In addition, GrM was usually co-expressed with PFN.

We studied GrM expression by $CD4^+$ and $CD8^+$ T cells, and compared this to their GrB and PFN expression. GrM was expressed in about 50 percent of $CD8^+$ T cells and in about only four percent of $CD4^+$ T cells (Figure 2A and Table 2). Despite the relative small shift in intensity between GrM-negative and GrM-positive cells, analysis for co-expression with GrB demonstrated that, in $CD8^+$ T cells, about 90 percent of GrM-positive cells also expressed GrB Although somewhat less explicit, such a concordance was also seen for GrM and PFN (about 60 percent). For the total population of $CD8^+$ T cells, GrM was co-expressed with GrB and PFN in about 40 and 30 percent of the cells, respectively (Figure 2A). GrM protein expression was absent in non-T non-NK cells in PBMC. Thus, $CD8^+$ T cells that express the cytotoxic proteins GrB and PFN in general also express GrM, and CD4+ T cells hardly express any of these three proteins.

GrM expression in specific T cell subsets

Next, we determined which specific cellular subsets in the $CD8^+$ T cell population expressed GrM. Six-colour flow cytometry analysis was performed for GrM and several cell membrane markers on PBMC from three healthy adult donors. In the $CD8^+$ T cell population, 40 percent of both the naive $CD27^+$ $CD45RO^-$ and early effector memory $CD27^+$ $CD45RO^+$ T cells (P4 and P5, respectively) expressed GrM (Figure 2B + Figure 3A). The more differentiated effector memory $CD27^-$ $CD45RO^+$ and effector $CD27^-$ $CD45RO^-$ T cells (P6 and P7, respectively) contained a higher percentage of GrM-positive cells, which gradually increased up to 80 ± 9 percent (Figure 3A). Analysis of the various $CD4^+$ T cell subsets also showed an increase in GrM expression upon differentiation, although the percentages


A
SSC
FSC
P1
CD8
CD3
P2
P3
GrB
GrM
P2 14.4 40.6 5.7
P3 3.7 2.9 0.9
PFN
GrB
P2 6.0 33.0 22.5
P3 4.4 5.2 2.0
PFN
GrM
P2 10.4 30.1 19.2
P3 7.2 2.6 1.5
B
CD8
CD3
P2
CD45RO
CD27
P6 P5 P7 P4
Counts
GrM
P6 47.1
P5 28.1
P7 65.6
P4 29.7

◄ ***Figure 2. Protein expression of GrM, GrB, and PFN by lymphocytes.*** *A) Analysis of GrM, GrB, and PFN protein expression in $CD3^+CD8^+$ (P2) and $CD3^+CD4^+$ (P3) T cells by flow cytometry, using PBMC from a healthy donor. The percentages of cells in each quadrant are shown in the upper right corners. Data represent one out of three experiments with similar results. B) Flow cytometry analysis of GrM expression by the four indicated subsets (P4-7) using anti-GrM AF488-conjugated mAb 4B2G4. $CD3^+CD8^+$ cells (P2) were sub-divided using the markers CD27 and CD45RO, discriminating a $CD27^+CD45RO^-$ (P4), $CD27^+CD45RO^+$ (P5), $CD27^-CD45RO^+$ (P6), and $CD27^-CD45RO^-$ (P7) subset. The percentage of GrM-positive cells is shown in the upper right corners. Data represent one out of three experiments with similar results.*

of cells that expressed GrM were much lower than for $CD8^+$ T cells (Figure 3A). $CD4^+$ $CD27^-$ $CD45RO^-$ T cells were not included because of their very small proportion in healthy donors. Thus, the GrM protein expression pattern suggested that expression of this protease is gradually induced during differentiation of T cells.

To confirm the differences in GrM protein expression, we compared the fraction of cells that expressed GrM protein with the GrM mRNA level for each cellular subset. The diverse $CD8^+$ and $CD4^+$ sub-populations and NK cells were sorted from PBMC of two healthy donors and their RQ of GrM mRNA were analysed by RT Q-PCR. In analogy with GrM protein expression, purified $CD8^+$ T cells and NK cells showed abundant levels of GrM mRNA as compared to $CD4^+$ T cells (Figure 3A). Of the $CD8^+$ T subsets, $CD27^-$ $CD45RO^-$ cells also displayed the highest RQ for GrM mRNA expression. However, in contrast to its protein expression, no gradual increase of GrM mRNA was observed in the sequential differentiating $CD8^+$ subsets (Figure 3A). Analysis of RQ of PFN and GrB mRNA in the sorted cell populations showed that these two mRNA patterns were both similar to GrM, in that NK cells and differentiated effector $CD8^+$ $CD27^-$ $CD45RO^-$ T cells expressed the highest mRNA levels of GrM, GrB, and PFN (Figure 3A+B). Taken together, the GrM protein expression pattern in T cell subsets as determined by flow cytometry analysis was confirmed by RT Q-PCR analysis and showed that GrM is highly expressed by effector $CD8^+$ $CD27^-$ $CD45RO^-$ T cells as well as NK cells.

Regulation of GrM protein expression

To study the regulation of GrM protein expression by the various lymphocyte subsets, PBMC of two healthy donors were stimulated with a panel of nine cytokines, either alone or in combination with anti-CD3. Cells were harvested after three and six days of culture with the various (combinations of) stimuli, followed by flow cytometry analysis of GrM expression by NK cells, NKT cells, γδ T cells, as well as GrM and GrB expression by $CD8^+$ and $CD4^+$ T cells. Both the percentage of GrB-positive $CD8^+$ T cells and the intensity of GrB expression by $CD8^+$ T cells were dramatically increased by all stimuli except for rh-IL-6, indicating functionality of the cytokines, *e.g.* rh-IL-2 (Figure 4A, left panels). In contrast, none of the single cytokines showed a significant change in fractions of GrM expressing cells after three or six days, neither for NK cells and $CD8^+$ T cells (t = 6 days in Figure 4B), nor for the other tested cellular subsets (results not shown). Incubation with rh-IL-12 resulted in a slightly higher percentage of GrM expressing NK cells as compared to non-stimulated cells after six days, however, this was not significant (p=0.34; t-test). A combination of anti-CD3 with one of the nine other stimuli or anti-CD3 alone resulted in a slight but non-significant decrease in the fraction of GrM expressing cells within the $CD8^+$ T cells population (results not shown). These data indicate that GrM protein expression is differentially regulated as compared to GrB.

Table 2. Overview of the GrM, GrB, and perforin protein expression by lymphocyte subsets. *The expression of the three granule-associated proteins per cellular subset is represented by the percentage of positive cells and the intensity of protein expression (+: intermediate, ++: strong). n.d.: not determined.*

Cellular subset	GrM		GrB		Perforin	
	%	Intensity	%	Intensity	%	Intensity
NK cells ($CD3^-CD56^+$)	81	+	n.d.	n.d.	76	+
NKT cells ($CD3^+CD56^+$)	72	+	n.d.	n.d.	66	+
γδ T cells ($CD3^+\gamma\delta^+$)	82	++	n.d.	n.d.	n.d.	n.d.
$CD8^+$ T cells	49	+	55	++	40	+
$CD4^+$ T cells	4	+	7	++	10	+

Discussion

In the present study, we demonstrate that human GrM is highly expressed by NK cells, NKT cells, γδ-T cells, and differentiated effector $CD8^+$ $CD27^-$ $CD45RO^-$ T cells. GrM protein expression was investigated by flow cytometry analysis using a novel specific mAb (4B2G4). Double-staining of $CD8^+$ T cells with GrM and either GrB or PFN revealed a high concordance in the cellular expression of these cytotoxic proteins (Figure 2A), whereas cross-reactivity with GrB was excluded (Figure 1A). The fractions of cells in the diverse lymphocyte subsets that expressed GrM protein were confirmed by RT Q-PCR analysis for GrM mRNA levels in purified sub-populations (Figure 3A). These results show that our novel mAb 4B2G4 is specific and sensitive to detect cellular GrM protein expression by flow cytometry analysis.

The observed expression of GrM in $CD8^+$ T cells in the present study is in agreement with Bade *et al.* [15], but in contrast to Sayers *et al.* [16]. The latter one determined only GM expression by NK cells, NKT cells, and γδ T cells, and not by $CD8^+$ T cells [16]. Like Bade *et al.* [15], we now demonstrated that GrM is also expressed by $CD8^+$ T cells, besides the three cell types of the innate immune system. The use of a different anti-GrM mAb in all three studies probably underlies the observed discrepancy. We and Bade *et al.* both generated and selected a mAb that recognizes native GrM and can be used for flow cytometry analysis, whereas Sayers *et al.* analysed GrM expression by western blotting of sorted cell populations, using a mAb that only detects denatured GrM [15,16]. Compared to western blotting, flow cytometry analysis can detect GrM expression at the single cell level. We showed that, in $CD8^+$ T cells, GrM was highly expressed only by the $CD27^-$ $CD45RO^-$ subset (Figure 2B + Figure 3A), which represents a small fraction of all T cells in healthy donors. This small amount might be undetectable by western blot analysis of sorted $CD8^+$ T cells, as applied by Sayers *et al.* [16]. So, using our novel mAb, we confirmed expression of GrM by $CD8^+$ T cells, as previously reported by Bade *et al.* [15], and in addition, showed that GrM protein expression is highest in the phenotypically most differentiated T cells. Whether GrM is also expressed by non-cytotoxic lymphoid cells or even myeloid cell types and exert PFN-independent functions, like GrB [22,23], requires further investigation. We observed, however, no GrM protein expression by non-T non-NK cells in PBMC.

Based upon its expression by NK cells, NKT cells, γδ-T cells, GrM was initially suggested to play a role in the innate immune response [16,24,25]. Analysis of GrM-deficient mice showed that these mice have an increased susceptible to murine cytomegalovirus infection, but normal NK cell-mediated tumor rejection [26]. Mice, however, harbour 11 instead of five granzymes,

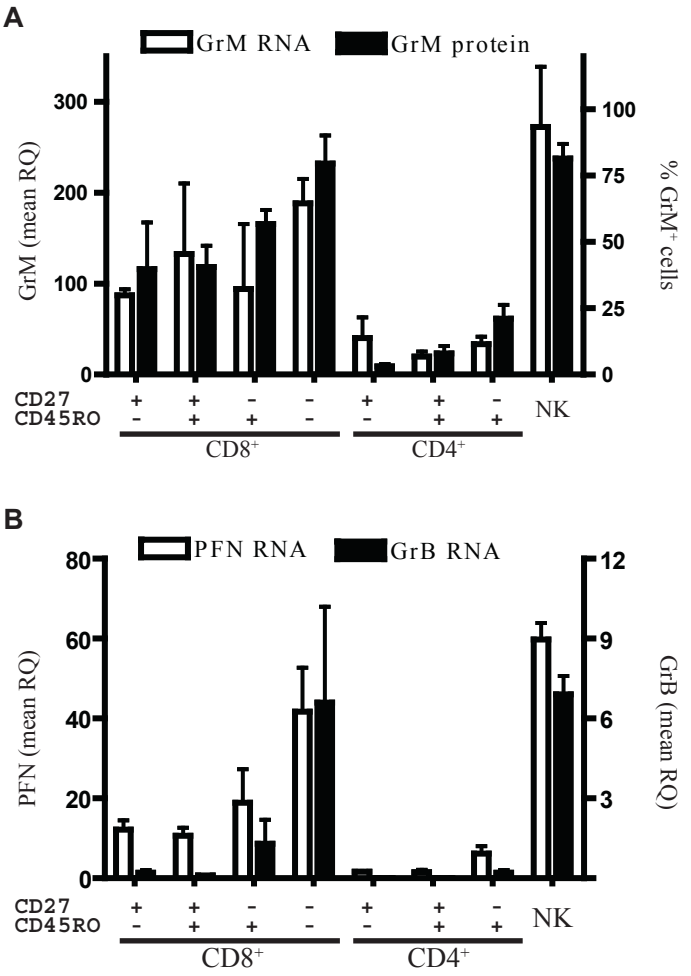


Figure 3. Comparison of mRNA and protein expression of GrM in T cell subsets. *A) mRNA (white bars; left y-as) and protein (black bars; right y-as) expression of GrM by NK cells and the indicated CD8⁺ and CD4⁺ T cell subsets. For mRNA, data represent the mean RQ / sorted sub-population (± SD) of two individual experiments performed in duplicate, as determined by RT Q-PCR. mRNA extracted from KHYG-1 was used as calibrator sample and GAPDH as reference gene. Protein data represent the mean percentage of GrM-positive cells / sub-population (± SD) of three individual experiments (including the two donors used for cell sorting and RT Q-PCR analysis), as determined by flow cytometry on PBMC using AF488-conjugated mAb 4B2G4. B) mRNA expression levels of PFN and GrB by NK cells and the indicated CD8⁺ and CD4⁺ T cell subsets, determined and represented similarly as for GrM in Figure 3A.*

as compared to humans, suggesting more redundancy between their granzymes. In addition, evidence for differences in substrate-specificity between the human and murine orthologue granzymes is emerging [27-29]. In line with this proposed redundancy, the cytotoxic potential of CTL from GrB- and GrA/B-deficient mice was not impaired and these mice displayed a normal rejection of tumor cells [30,31], suggesting a cytotoxic role for the orphan granzymes.

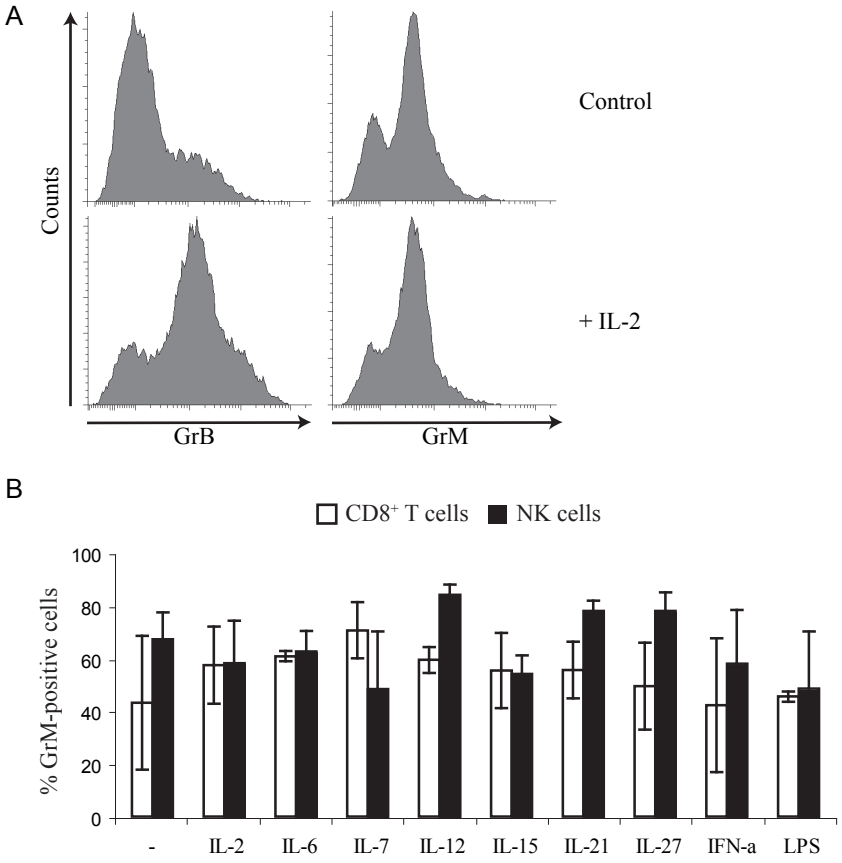


Figure 4. Regulation of GrM protein expression by CD8⁺ T cells and NK cells. *A) GrB and GrM expression by CD8⁺ T cells after 6 days of culture of PBMC without stimuli (upper panels) or with rh-IL-2 (lower panels). Histograms are an example of all tested stimuli (except rh-IL-6) and represent data from one out of two donors with similar results. B) Percentages of GrM-positive CD8⁺ cells and NK cells after culture of PBMC for six days with the indicated stimuli. Data represent mean (+/- SD) of two independent experiments.*

Together with our current confirmation of the finding by Bade *et al.* [15] that GrM is also expressed by CD8⁺ T cells (Figure 2A + Table 2), this points to a role for human GrM in the adaptive immune response. As a consequence, previous studies based upon the assumption that GrM is only expressed by innate immune cells probably require some re-interpretation. Based upon the finding that almost all cases of various types of lymphomas expressed GrM, it was suggested that these tumors originate from NK cells, NKT cells, and γδ-T cells [25,32]. However, such lymphomas may thus also originate from GrM-expressing CD8⁺ T cells, which has been previously suggested by de Bruin *et al.* based upon the expression of GrB [33].

Using CD27 and CD45RO as markers to discriminate for the various T cell subsets representing distinct differentiation stages, we observed a gradual increase in the protein expression of GrM from naive T cells, via effector-memory cells, to effector cells (Figure 2B + 3A). Previous studies on protein expression of human GrA, GrB, and PFN also showed

a gradual increase in the percentages of cells that expressed either one of these cytotoxic proteins during T cell differentiation [34,35]. In contrast, GrK was differentially expressed as compared to GrA and GrB. Its expression was absent in more differentiated memory $CD8^+$ T cells, as determined by markers like CD27, CD28, CCR5, and CCR7 [9]. GrK expression has also been suggested not to associate with the cytotoxic potential of lymphocytes [15]. Our current findings suggest that expression of GrM may be important for the cytotoxic potential of memory as well as effector $CD8^+$ T cells. Interestingly, GrM mediates a unique cell death pathway, characterised by swelling of a target cell and vacuolisation [8]. In addition, the GrB-inhibitor SERPINB9, which is over-expressed by several types of tumors and associates with poor clinical outcome [36,37], is cleaved by GrM *in vitro*, suggesting that GrM can overcome SERPINB9-mediated inhibition of GrB and thereby clears the way for efficient GrB-induced target cell death [38].

In contrast to its cellular expression pattern, GrM seems to be differentially regulated by cytokines compared with GrB. The percentage of $CD8^+$ T cells that expressed GrB increased and the protein expression level of GrB per cell was up-regulated by most cytokines. In contrast, GrM expression was not significantly affected by either one of the tested combinations of stimuli. This finding is in line with a report by Zhang *et al.*, in which they described that none of the cytokines IL-2, IL-12, IL-15, or IFN-α had an effect on GrM mRNA expression by the NK cell line NK-92 [39]. Thus, it remains to be elucidated which stimuli can affect GrM expression. This may be of clinical importance in the future, since a drug-mediated increase in GrM expression by lymphocytes may lead to enhanced cytotoxicity against tumor and/or virus-infected target cells. The differential expression and regulation of the five human granzymes might be explained by the distinct chromosomal localizations of their genes. The genes encoding GrA and GrK are clustered on chromosome 5, the GrB and GrH locus reside at chromosome 14, and GrM is encoded by a gene on chromosome 19 [6]. On the other hand, granzymes of the same locus are also expressed discordantly [9,11,15], suggesting unique binding sites for specific transcription factors in the promotor region of each granzyme gene. So far, this has only been studied for human GrA and GrB [5].

In conclusion, the human cytotoxic protease GrM is, besides by innate immune cells, also expressed by $CD8^+$ effector T cells, suggesting not only a role for GrM in the innate but also in the adaptive immune response.

Acknowledgements

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Chapter 5

NK cell protease granzyme M targets α-tubulin and disorganizes the microtubule network

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Abstract

Serine protease granzyme M (GrM) is highly expressed in the cytolytic granules of natural killer (NK) cells, which eliminate virus-infected cells and tumor cells. The molecular mechanisms by which GrM induces cell death, however, remain poorly understood. Here, we employed a proteomic approach to scan the native proteome of human tumor cells for intracellular substrates of GrM. Among others, this approach revealed several components of the cytoskeleton. GrM directly and efficiently cleaved the actin-plasma membrane linker ezrin and the microtubule component α-tubulin, using purified proteins, tumor cell lysates, and tumor cells undergoing cell death induced by perforin and GrM. These cleavage events occurred independent of caspases or other cysteine proteases. Kinetically, α-tubulin was more efficiently cleaved by GrM as compared to ezrin. Direct α-tubulin proteolysis by GrM is complex and occurs at multiple cleavage sites, one of them being Leu at position 269. GrM disturbed tubulin polymerization dynamics *in vitro* and induced microtubule network disorganization in tumor cells *in vivo*. We conclude that GrM targets major components of the cytoskeleton, which likely contribute to NK cell-induced cell death.

Abbreviations

Gr	Granzyme
MS	Mass spectrometry
PI	Propidium iodide.

Introduction

Cytotoxic lymphocytes, *i.e.* cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, are key players in the effector arm of the immune response that eliminates virus-infected cells and tumor cells [1,2]. Cytotoxic lymphocytes predominantly destroy their targets by releasing the content of their cytolytic granules. These granules contain perforin and a family of unique structurally homologous serine proteases known as granzymes [3,4]. While perforin facilitates the entry of granzymes into the target cell, the latter induce cell death by cleaving critical intracellular substrates [1,2].

In humans, five different granzymes (GrA, GrB, GrH, GrK, and GrM) are known that differ on the basis of their substrate specificity [3,4]. Over the past few decades, it has been well established that GrA and GrB serve as important determinants of cellular cytotoxicity. Both granzymes induce nuclear and non-nuclear damage in target cells, by cleaving distinct non-overlapping sets of substrates [1,2]. Two important intracellular substrates of GrB include pro-caspase 3 [5] and the small BH3-only protein Bid [6]. Cleavage of these proteins leads to DNA fragmentation and mitochondrial damage, respectively. GrA predominantly kills by cleaving nuclear (e.g. Ku70), mitochondrial, and cytoplasmic substrates (e.g. SET complex components) [2,7-9]. Cleavage of these substrates results in single-stranded nicking of chromosomal DNA.

In contrast to GrA and GrB, far less is known about the other human granzymes. It has been demonstrated that GrM, which is specifically expressed by NK cells, mediates a novel major and perforin-dependent cell death pathway with unique morphological hallmarks that plays a significant role in NK cell induced death [10]. The molecular mechanism by which GrM induces cell death remains unclear. One study has found that GrM-induced cell death occurs independent of caspases, DNA fragmentation, and reactive oxygen species (ROS) generation [10], whereas other recent reports have demonstrated the opposite [11,12]. This suggests that GrM targets multiple independent cell death pathways, which has also been demonstrated for GrA and GrB [1,2,5-9]. In the present study, we employed a proteomic approach to define potential substrates of GrM. We report that GrM targets the cytoskeleton in tumor cells by cleaving the actin-plasma membrane linker ezrin and the microtubule component α-tubulin. This likely contributes to the mechanism and the specific morphological changes that coincide with GrM-mediated target cell death.

Materials and Methods

Reagents

Antibodies were anti-α-tubulin clone B-5-1-2 (Sigma), anti-ezrin clone 3C12 (Zymed Laboratories), anti-β-actin clone 2A2.1 (US Biological), anti-caspase-3 clone H-277 (Tebu-bio), anti-GST tag (Santa Cruz Biotechnology), and anti-His tag (BD Biosciences). E64 [trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane] was from Sigma, and z-VAD-fmk [Z-Val-Ala-Asp(OMe)-Fluoromethylketone] was from Biomol. The chromogenic caspase-3 substrate Ac-Asp-Glu-Val-Asp-pNA (Ac-DEVD-pNA) was from Bachem. Purified recombinant human GST-k-α-1-tubulin was purchased from Cytoskeleton. Human perforin was purified as described [13]. Protein was quantified by the method of Bradford.

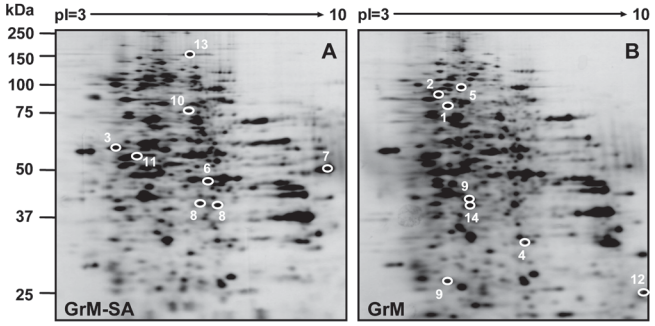

Figure 1. Identification of GrM-induced cleavage events in tumor cell lysates. *HeLa cell freeze/thaw lysates were incubated with GrM-SA (1 μM) (A) or GrM (1 μM) (B) for 60 min at 37°C. Proteins were separated by 2D gel electrophoresis (10%) and visualized by silver staining. Proteins that are reduced in abundance after GrM incubation represent potential GrM substrates (A) and new spots that appear during GrM treatment are cleavage products (B). This experiment was performed 3 times with similar results and changed protein spots (n = 37) were excised from 2D gels. Protein spots that could be identified (n = 16) by LC-MS/MS are indicated by white circles.*

Table I. Overview of GrM-induced cleavage events identified by tandem MS. *Spot numbers correspond to the numbered spots in gel images of Fig. 1. Protein details were assessed in the Swiss-Prot database.*

Spot nr.	*Protein identity*	*Swiss-Prot. accession number*	*Theoretical mol. mass / pI details*
	Chaperones / cell stress response		
1	Heat shock protein 90β (HSP90β)	P08238	83.1 / 5.0
2	Endoplasmin (Grp94)	P14625	92.5 / 4.8
3	Protein disulfide-isomerase (PDI)	P07237	57.1 / 4.8
	Translation machinery		
4	Heterogeneous nuclear ribonucleoproteins (hnRNP) A2/B1	P22626	37.4 / 9.0
5	116 kDa U5 small nuclear ribonucleoprotein component	Q15029	109.4 / 4.8
6	Elongation factor Tu (EF-Tu)	P49411	49.5 / 7.3
7	Elongation factor 1-α-1 (EF-1-α-1)	P68104	50.1 / 9.1
8	Poly(rC) binding protein 1 (hnRNP E1)	Q15365	37.5 / 6.7
	Cytoskeleton		
9	β-Actin	P60709	41.7 / 5.5
10	Ezrin	P15311	69.3 / 6.0
11	α-Tubulin	Q71U36	50.1 / 4.9
	Miscellaneous		
12	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	P00354	35.9 / 6.6
13	Carbamoyl-phosphate synthase (CPSase I)	P31327	164.9 / 6.3
	Less defined function		
14	130 kDa Leucine-rich protein (LRP130)	P42704	145.2 / 5.5

Cell lines and cell-free protein extracts

HeLa and Jurkat cells were grown in DMEM and RPMI 1640 medium, respectively, supplemented with 10% fetal calf serum (FCS), 0.002 M glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Cell-free protein extracts were generated from exponentially growing HeLa and Jurkat cells. Cells (10^8 cells/ml) were washed two times in a buffer containing 50 mM Tris (pH 7.4) and 150 mM NaCl, and lysed in the same buffer by three cycles of freeze-thawing. This method gently disrupts the plasma membrane and minimally affects cell compartment integrity [14]. Samples were centrifuged for 10 min at 14,000 rpm at 4°C and cell-free protein extracts were stored at -80°C.

Recombinant proteins

The cDNA encoding mature human GrM (residues Ile^{26}-Ala^{257}) was amplified from IMAGE clone 5222281 and cloned into yeast expression vector pPIC9 (Invitrogen). Catalytically inactive GrM-SA, in which Ser^{195} residue in the catalytic center is replaced by Ala, was generated by site-directed mutagenesis (Stratagene). Plasmids were transformed into the GS115 (his4) strain of P. pastoris and granzymes were expressed in conditioned media for 72 h as described by the manufacturer (Invitrogen). GrM and GrM-SA were purified to homogeneity by cation-exchange chromatography (GE Healthcare), using a linear salt gradient for elution. GrM preparations were dialyzed against 50 mM Tris (pH 7.4) and 150 mM NaCl, and stored at -80°C. GrM, but not GrM-SA, was active as determined by a synthetic chromogenic leucine substrate (Bachem) (data not shown).

The human k-α-1-tubulin cDNA was amplified from IMAGE clone 3871729, cloned into the bacterial expression vector pQE80L, and expressed as recommended by the manufacturer (Invitrogen). The L269A, L286A, L286A/L269A, M302A, M302A/L269A, M313A, M313A/L269A, and L317A/L318A tubulin mutants were generated by site-directed mutagenesis. Recombinant His-α-tubulin protein was purified by metal-chelate chromatography (Clontech), dialyzed against phosphate-buffered saline (PBS), and stored at -80°C. Ezrin cDNA was from RZPD German Resource Center for Genome Research. The pGEX-GST-Ezrin bacterial expression construct, in which the GST tag is fused to the N-terminus of human ezrin, was kindly provided by dr. H. Rehmann (UMC Utrecht, The Netherlands). Recombinant GST-ezrin was expressed and purified as described above for recombinant His-α-tubulin.

2D gel electrophoreses and spot identification by mass spectrometry

Washed HeLa cells (10^8 cells/ml) in 25 mM Tris (pH 8), 30 mM NaCl, and 1 mM dithiothreitol (DTT), were subjected to three rounds of freeze/thaw lysis and cell free extracts (50 µg) were incubated with GrM (1 µM) or GrM-SA (1 µM). After 1 h at 37°C, samples were precipitated using the Plus One 2D Clean-up kit as recommended by the manufacturer (GE Healthcare) and solubilized in 8 M urea, 2 M thiourea, 4% Chaps, 20 mM DTT, 0.2% biolyte pH 3-10, and 0.2% bromophenol blue (200 µl) for iso-electric focusing. Samples (50 µg) were re-hydrated passively into 11 cm pH 3-10 IPG strips for 15 h at RT prior to iso-electric focusing in the IPGphor system (GE Healthcare) for 20 kVh. IPG strips were reduced for 60 min in 2% (w/v) DTT, 6 M Urea, 2% (w/v) SDS, 20% (v/v) glycerol, 0.375 M Tris, pH 8.8, and alkylated for 30 min in the same buffer containing 2% (w/v) iodocetamide instead of DTT. Strips were mounted on 10% SDS-PAGE gels, proteins were separated, and 2D gels were stained by MS-compatible silver staining. Gel features were evaluated by PDQuest 7.4 software and selected spots were excised robotically with a ProteomeWorks Spot Cutter

(Biorad). Gel cores were destained and subjected to in-gel tryptic digestion. Peptide mixtures were applied to LC-MS/MS (Finnigan LTQ) and the results were analyzed by MASCOT (www.matrixscience.com).

GrM-mediated cell death

Washed Jurkat cells (1 x 10^6) were treated with GrM (1 μM) or GrM-SA (1 μM) in the presence or absence of a sublytic dose of perforin (40 ng/ml) in 50 mM Hepes, pH 7.4, 150 mM NaCl, 2.5 mM $CaCl_2$, 1% (w/v) bovine serum albumin (BSA) for 4 or 10 h at 37°C. Cells were washed in the same buffer and used for cytospins, PI flow cytometry, or direct lysis with SDS-PAGE loading buffer. Cytospins were fixed with 96% (v/v) ethanol for 10 min and stained with Giemsa or immuno-stained with an antibody against α-tubulin (clone B-5-1-2), followed by a TRITC conjugated antibody to visualize α-tubulin by confocal microscopy. For flow cytometry, cells were incubated with PI (46 μg/ml) for 10 min at RT. Cell viability after a 24 h-incubation period with GrM/perforin or GrM-SA/perforin was measured by trypan blue staining.

Tubulin polymerization assay

A tubulin polymerization assay kit (Cytoskeleton) was employed to address the effects of GrM on tubulin polymerization dynamics. A purified bovine α- and β-tubulin preparation (3 mg/ml), virtually free of microtubule-associated proteins (MAPs) (Cytoskeleton, HTS02), in 80 mM Pipes, pH 6.9, 0.5 mM EGTA, 2 mM $MgCl_2$ was incubated with GrM (1 μM), GrM-SA (1 μM), Paclitaxel (taxol) (5 μM), or buffer in 20 mM Tris, pH 7.0. With the exception of taxol, samples were pre-incubated for 2 h at 30°C. Microtubule polymerization was initiated by the addition of GTP (1 mM) and 5% (v/v) glycerol. Changes in microtubule turbidity were measured kinetically at 340 nm at 37°C (Anthos Labtec).

Results

GrM-induced cleavage events in tumor cell lysates

To define potential intracellular substrates of GrM we employed a protease-proteomic approach. Because it is difficult to deliver large amounts of GrM to all target cells via perforin, we used freeze/thaw lysis of HeLa tumor cells to gently disrupt the plasma membrane and minimally alter the native proteome. These protein extracts were incubated with purified recombinant mature human GrM or the catalytically inactive GrM-SA mutant and cleavage events were analyzed by 2D-gel electrophoresis (Fig. 1). Spots present in greater abundance in the control sample indicate possible GrM substrates, whereas spots present in greater abundance in the GrM-treated sample reflect potential cleavage products. Of approximately 1500 proteins that were resolved in this proteomic screen, about 15 spots clearly disappeared (Fig. 1A) and about 22 spots clearly appeared (Fig. 1B) following the incubation with GrM. These changes were highly reproducible and could also be detected when samples were labeled fluorescently and analyzed on the same 2D gel, using fluorescence 2D difference gel electrophoreses (fl-2D-DIGE) (data not shown).

Spots were excised that exhibited high reproducibility and displayed greater than 3-fold changes in abundance following GrM treatment. We were able to identify 16 of 37 excised protein spots from 2D gels, employing tandem mass spectrometry (MS) (Table I). Several

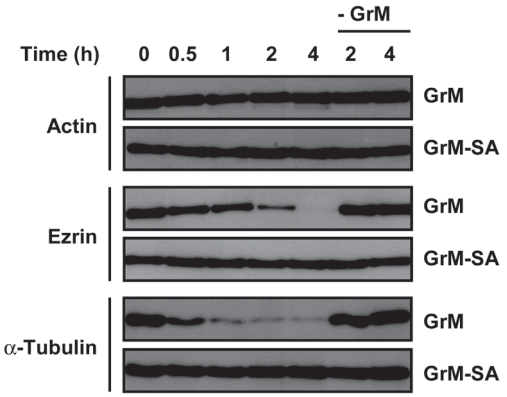

Figure 2. Ezrin and α-tubulin are cleaved by GrM in tumor cell lysates. *HeLa cell lysates were incubated with GrM (1 μM), GrM-SA (1 μM), or buffer for indicated times at 37°C. Samples were immunoblotted, using antibodies against β-actin, ezrin, and α-tubulin. These experiments were repeated at least 3 times with the same results.*

spots that consistently changed following GrM treatment could not be identified, most likely because protein levels were too low. The identity of potential GrM substrates that could be identified include a group of highly homologous proteins involved in chaperone systems and cellular stress response (i.e. HSP90β, Endoplasmin, and PDI), proteins involved in translational machinery (i.e. hnRNP A2/B1, U5 snRNP component, EL-Tu, EL-1-α-1, and hnRNP E1), a protein of less well defined function (i.e. LRP130), some miscellaneous (i.e. GAPDH and CPSase I), and several components that control the integrity of the cytoskeleton (i.e. β-actin, ezrin, and α-tubulin) (Table I).

GrM cleaves cytoskeleton-related proteins

The group of proteins that control the integrity of the cytoskeleton (i.e. β-actin, ezrin, and α-tubulin) were selected for further studies, because inactivation or down-regulation of these proteins has been causally linked to cell death and are known to protect tumor cells from apoptosis [15-19]. In addition, they reside in the cytoplasm and as such are accessible for GrM. Finally, α-tubulin has recently been identified as a physiological substrate of the cytotoxic lymphocyte component GrB [15,20] and α-tubulin currently constitutes among the most successful target for anticancer chemotherapy [18,19]. To verify cleavage of β-actin, ezrin, and α-tubulin by GrM in protein extracts, HeLa cell lysates were incubated in the presence or absence of GrM or GrM-SA and subjected to immunoblotting, using antibodies against these proteins (Fig. 2). Incubation of lysates with GrM resulted in time-dependent cleavage of ezrin and α-tubulin, but not β-actin. Unlike ezrin and α-tubulin, the β-actin protein was identified by tandem MS from cleaved β-actin fragments that appeared during GrM cleavage (spot numbers 9 in Fig. 1B). This precludes information on the efficiency by which intact β-actin is cleaved by GrM. Western blot analysis, however, now indicates that the bulk of intact β-actin protein remains uncleaved (Fig. 2).

Although the monoclonal antibodies used could not detect cleavage products, GrM-mediated cleavage of ezrin and α-tubulin was illustrated by the progressive time-dependent disappearance of both ezrin and α-tubulin protein bands. The molecular weights of non-

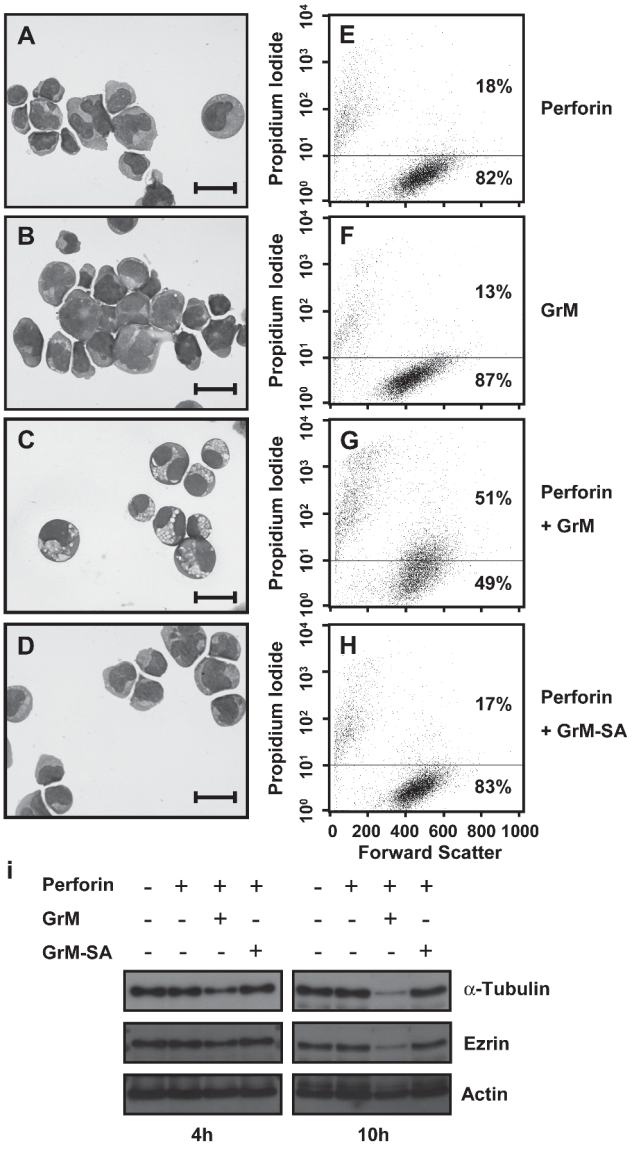

Figure 3. Ezrin and α-tubulin are cleaved by GrM in tumor cells undergoing cell death. *A-D, Jurkat cells (1 x 10^6) were treated with a sublytic dose of perforin (40 ng/ml) and GrM (1 μM), perforin (40 ng/ml) and GrM-SA (1 μM), perforin (40 ng/ml) alone, or GrM (1 μM) alone for 4 h at 37°C. Cells (1 x 10^5) were centrifuged on glass slides and visualized by Giemsa staining. Bar, 20 μm. E-H, Cells (1 x 10^4) were incubated with PI (1 μg/ml) for 10 min at RT and analyzed by flow cytometry. I, At indicated time points, cells were lysed and whole cell protein extracts were immunoblotted, using antibodies against α-tubulin, ezrin, and β-actin. These experiments were repeated at least 3 times with the same results.*

cleaved ezrin and α-tubulin matched with the molecular weights of the protein spots excised from GrM-SA-treated lysates on 2D gels (Fig. 1A) and to that of the theoretical molecular mass of these proteins (Table I). Cleavage of α-tubulin by GrM in cell lysates was virtually

completed after 30-60 min of incubation, whereas complete cleavage of ezrin occurred after 2-4 h. GrM-SA did not show any reactivity with these substrates, indicating that all cleavage events are specific for GrM proteolytic activity. Similar results were obtained when Jurkat cell lysates were employed (data not shown). These data indicate that ezrin and α-tubulin are direct or indirect substrates of human GrM and that α-tubulin kinetically was more efficiently cleaved in tumor cell lysates as compared to ezrin.

GrM cleaves α-tubulin and ezrin in tumor cells undergoing cell death

To examine whether α-tubulin and ezrin are also cleaved in intact cells undergoing cell death induced by GrM, we employed a cell death assay in which perforin is used to deliver GrM into the tumor cell (Fig. 3). It has been established that GrM-induced cell death can be detected by typical morphological changes and by measuring membrane integrity by propidium iodide (PI) flow cytometry [10]. Consistent with this, GrM/perforin-treated cells demonstrated the morphological changes as described [10], such as signs of chromatin condensation and the presence of large cytoplasmic vacuoles (Fig. 3C). As expected [10], GrM/perforin-treated cells showed higher PI staining as compared to controls (Fig. 3E-H). Lysates of GrM- or GrM-SA-treated cells were subjected to immunoblotting and cleavage of α-tubulin and ezrin was monitored (Fig. 3I). Cleavage of α-tubulin or ezrin was not observed in cells treated with buffer, perforin alone, or perforin in combination with GrM-SA, but was detected in cells treated with perforin and GrM. After 4 h of incubation, a modest reduction of α-tubulin could be observed, whereas only a minor reduction of ezrin was found. This is consistent with the observed differential cleavage kinetics of both proteins by GrM (Fig. 2). Cleavage of α-tubulin and ezrin, however, was nearly complete after a 10 h incubation period. In line with the results obtained in Fig. 2, no discernible hydrolysis of β-actin was observed. GrM-induced cell death was irreversible, as cell viability of GrM/perforin-treated Jurkat cells was 2.1 (+/-2.0) % (mean +/- S.D.) as compared with 100 (+/-11.9) % for GrM-SA/perforin after a 24 h incubation period ($P < 0.001$). These results indicate that α-tubulin and ezrin, but not β-actin, are cleaved in tumor cells that undergo irreversible cell death induced by GrM in combination with perforin.

GrM cleaves α-tubulin and ezrin in a caspase-independent manner

Lu et al. [12] recently demonstrated that one way by which GrM induces cell death involves proteolytic activation of pro-caspase-3, whereas another study shows that GrM-mediated cell death completely occurs independent of caspases [10]. Therefore, we have addressed the role of caspases and other cysteine proteases during GrM-mediated proteolysis of α-tubulin and ezrin. To this end, we employed the pan-caspase inhibitor z-VAD-fmk and the broad spectrum cysteine protease inhibitor E64. Tumor cell lysates were incubated with GrM and proteolysis of α-tubulin and ezrin was monitored by immunoblotting (Fig. 4A). Neither z-VAD-fmk nor E64 affected GrM-mediated cleavage of α-tubulin and ezrin, indicating that neither caspases nor other cysteine proteases are involved in this process. E64 and z-VAD-fmk did not affect the activation of pro-caspase-3 by GrB (Fig. 4A), which is expected since GrB is a serine protease [1-4].

Next, we investigated the capability of GrM to cleave and/or activate pro-caspase-3. In contrast to Lu et al. [12], but consistent with Kelly et al. [10], GrM did not cleave pro-caspase-3 (Fig. 4A). Also at higher GrM concentrations and longer incubation times, GrM did not cleave this pro-caspase (Fig. 4B). In agreement with these findings, GrM did not activate

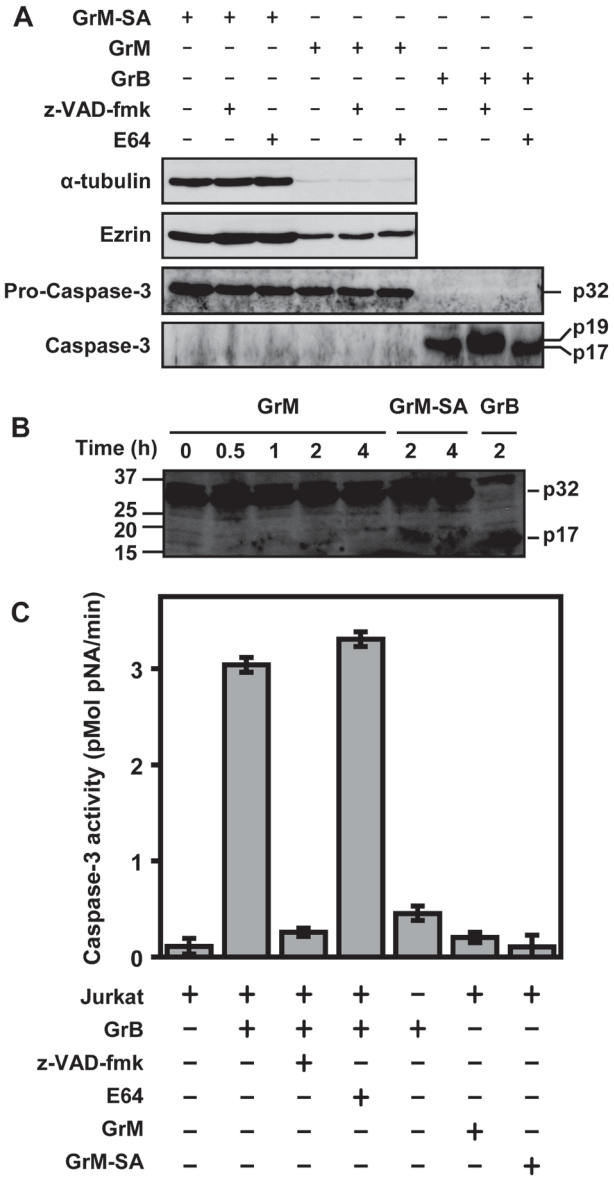

Figure 4. GrM cleaves α-tubulin and ezrin in a caspase-independent manner. *A, Jurkat cell lysates were incubated with GrM (1 μM), GrM-SA (1 μM), or GrB (1 μM) for 2 h at 37°C, in the absence or presence of z-VAD-fmk (10 μM) or E64 (10 μM). Samples were immunoblotted, using antibodies against α-tubulin, ezrin, or caspase-3. B, Jurkat cell lysates were incubated with GrM (2 μM), GrM-SA (2 μM), or GrB (0.5 μM) for 0-4 h at 37°C. Samples were immunoblotted, using antibodies against caspase-3. These experiments were repeated at least 3 times with the same results. C, Jurkat cell lysates were incubated with GrM (1 μM), GrM-SA (1 μM), or GrB (1 μM) for 4 h at 37°C, in the absence or presence of z-VAD-fmk (10 μM) or E64 (10 μM). Samples were incubated with the chromogenic caspase-3 substrate Ac-DEVD-pNA (0.5 mM) and measured kinetically at 405 nm for 60 min. Data are presented as pMol pNA that is cleaved from the chromogenic substrate per minute as mean +/- S.D. of three independent experiments.*

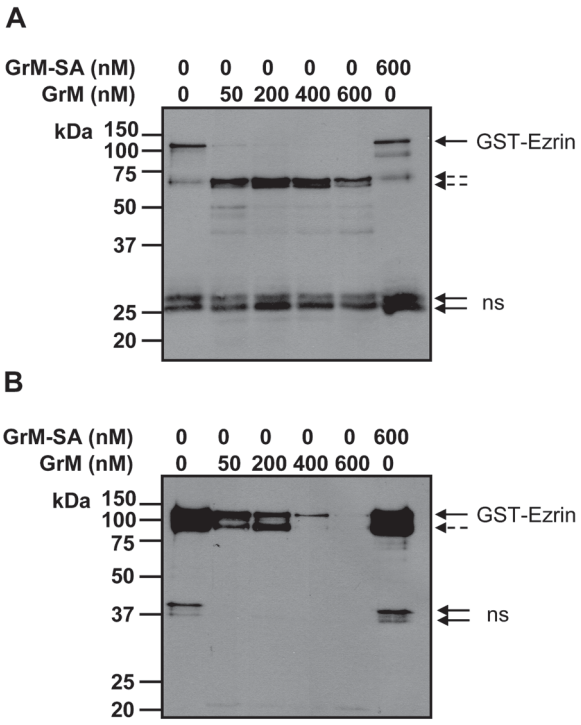

Figure 5. GrM directly cleaves ezrin. *A-B, Purified recombinant GST-ezrin (75 nM) was treated with indicated concentrations of GrM (0-600 nM) or GrM-SA (600 nM) for 2 h at 37°C. GrM:GST-ezrin stoichiometries were 1.5:1, 2.6:1, 5.3:1, and 7.7:1. Proteins were separated by SDS-PAGE (10%) and subjected to immunoblotting, using antibodies against the N-terminal GST tag (A) or the C-terminal part of ezrin (B). Full-length GST-ezrin (solid arrow) and cleavage products (dotted arrows) are indicated. ns, non-specific.*

pro-caspase-3 as determined by the small chromogenic caspase-3 substrate Ac-DEVD-pNA (Fig. 4C). GrB was included as positive control for cleavage and activation of pro-caspase-3 in these experiments. Indeed, the activity of caspase-3 was completely inhibited by the pan-caspase inhibitor z-VAD-fmk. As expected, E64 did not affect caspase-3 activity, given that E64 does not inhibit caspases [21]. E64 was functionally active since it efficiently blocked the cathepsin-binding properties of DCG-04, which is an active site-directed probe that is used to specifically label active cathepsin proteases (data not shown). Taken together, these results indicate that GrM neither cleaves nor activates pro-caspase-3 and that α-tubulin and ezrin are not cleaved by GrM in a caspase(-3)- or other cysteine protease-dependent manner.

GrM directly cleaves α-tubulin and ezrin

We investigated whether α-tubulin and ezrin constitute direct GrM substrates, rather than being the substrates of secondary proteases other than cysteine proteases or caspases present in tumor cells. To this end, GrM or GrM-SA was incubated with purified recombinant GST-α-tubulin or GST-ezrin. Treatment of purified GST-ezrin with GrM, but not GrM-SA, resulted in the disappearance of the expected about 100 kDa GST-ezrin protein band (Fig. 5). Using anti-GST (Fig. 5A) or anti-C-terminal ezrin antibodies (Fig. 5B), cleavage products

were detected by immunoblotting. This indicates that ezrin is a direct substrate of GrM. Whereas GrM-SA-treated and untreated GST-α-tubulin remained intact, treatment of purified α-tubulin with increasing concentrations of GrM resulted in the progressive disappearance of the approximately 75 kDa GST-fused α-tubulin protein and the appearance of two major cleavage products of about 52 kDa and 23 kDa (Fig. 6A). This cleavage event already occurred at low nanomolar concentrations of GrM (5-20 nM) and relatively high tubulin concentrations (1 μM). Immunoblotting with an antibody against GST revealed that the 52 kDa band represents the N-terminal α-tubulin moiety fused to the GST tag (data not shown). Higher concentrations of GrM further processed the N-terminal 52 kDa cleavage product, indicating that GrM cleaves α-tubulin at least at two sites.

A closer look at the molecular weights of the cleavage fragments of α-tubulin and knowing the proposed P1 primary and P2-P4 subsite specificities of GrM [22,23], we mutated the Leu residue at position 269 into Ala. Although this mutant His-tagged α-tubulin was also cleaved by GrM, a different amino-terminal cleavage product with increased molecular weight was observed (Fig. 6B). Using a highly sensitive fluorescent protein staining on GrM-cleaved wild-type and L269A mutant α-tubulin, the up-shift of the N-terminal proteolytic fragment of L269A α-tubulin mutant was again evident (Fig. 6C). Strikingly, however, under these conditions at least seven other α-tubulin cleavage fragments appeared when α-tubulin was cleaved by GrM (Fig. 6C). The L269A α-tubulin mutant kinetically was equally well cleaved by GrM as compared with wild-type α-tubulin (Fig. 6D), strongly suggesting that other GrM cleavage sites in α-tubulin are at least equally important. Furthermore, the N-terminal α-tubulin cleavage fragment appeared at low GrM concentrations with limited proteolysis at multiple sites until it completely disappeared when higher GrM concentrations are used (Fig. 6D).

We have attempted to identify additional GrM-cleavage sites by site-directed mutagenesis of Leu and Met residues more C-terminal of Leu^{269}. These α-tubulin mutants include L286A, L286A/L269A, M302A, M302A/L269A, M313A, M313A/L269A, and L317A/L318A. Except for L269A, we were not able to demonstrate a difference in proteolysis of these mutants, neither alone nor in combination with L269A (data not shown). This indicates that other Leu or Met residues in α-tubulin are more important GrM cleavage sites, or that GrM

Figure 6. GrM directly cleaves α-tubulin. *A, Purified recombinant GST-α-tubulin (1 μM) was treated with indicated concentrations of GrM (0-500 nM) or GrM-SA (500 nM) for 2 h at 37°C. GrM:GST-α-tubulin stoichiometries were 1:1000, 1:200, 1:50, 1:10, and 1:2. Proteins were separated by SDS-PAGE (10%) and stained with Coomassie Brilliant Blue. Full-length GST-α-tubulin (solid arrow) and cleavage products (dotted arrows) are indicated. B, Purified recombinant wild-type His-α-tubulin (WT) (1 μM) and the His-α-tubulin mutant in which Leu^{269} has been replaced by Ala (L269A) were treated with GrM (50 nM) or GrM-SA (50 nM) for 2 h at 37°C. Samples were subjected to Western blot analysis, using an anti-His tag antibody. C, Purified recombinant WT and mutant L269A His-α-tubulin (1 μM) were treated with or without GrM (50 nM) for 2 h at 37°C. Proteins were separated by SDS-PAGE (10%) and stained with fluorescent Flamingo staining. D, WT and mutant L269A His-α-tubulin were treated with GrM (0-500 nM) or GrM-SA (500 nM) for 2 h at 37°C Proteins were separated by SDS-PAGE (10%) and stained by immunoblotting, using an anti-His tag antibody. Full-length His-α-tubulin (solid arrow), and N-terminal (N1 and N2) cleavage products and other cleavage fragments (dotted arrows) are indicated (B-D). E, Schematic representation of α-tubulin domain structure, including the GrM cleavage site. F, Sequence alignment of amino acid region 246-289 of human α-tubulin isoforms. Amino acid identity is indicated in black, except that the GrM cleavage site is depicted in grey. A-D represent P1'-P4' and 1-4 represent P1-P4, respectively.* ►

A
GrM-SA (nM) 0 0 0 0 0 0 0 500
GrM (nM) 0 1 5 20 100 500 0
kDa 150 100 75 50 37 25 20
GST-α-tubulin
B
GrM-SA + - + - + -
GrM - + - + - +
WT
L269A
kDa 100 75 50 37 25
His-α-tubulin
N2
N1
C
GrM
WT
L269A
kDa 100 75 50 37 25
His-α-tubulin
N2
N1
GrM
D
GrM-SA (nM) 500 0 0 0 0 0 0 0 0 500 0 0 0 0 0 0 0 0 0 0 0
GrM (nM) 0 1 2 5 10 20 50 100 500 0 1 2 5 10 20 50 100 500
kDa 100 75 50 37 25
Wild-type α-tubulin
L269A α-tubulin
N2
N1
E
GrM
266HFPL↓ATYA273
N
C
0 GTP-binding domain 205 MAP-binding domain 451
F
246 I 4321ABCD 289
GALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVA α-Tubulin isoform-1
GALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVA α-Tubulin isoform-2
GALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVA α-Tubulin H2-α
GALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVA α-Tubulin-3
GALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVA α-Tubulin k-alpha-1
GALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVA α-Tubulin-1
GALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLTVA α-Tubulin-6
GALNVDLTEFQTNLVPYPRIHFPLVTYAPIISAEKAYHEQLSVA α-Tubulin-8
GPLNVDLIEFQTNLVPYPRIHFPMTAFAPIVSAEKAYHEQLSVS α-Tubulin-L3
GALNVDLTEFQTNLVSM------LTSTSPWP---PMHQSSLQKK α-Tubulin-4

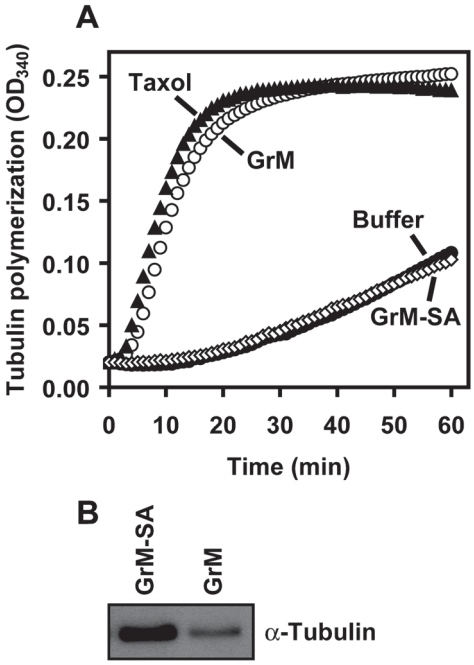

Figure 7. GrM disturbs tubulin polymerization dynamics. *A, Purified bovine tubulin (40 μM) was incubated with GrM (1 μM) (open circles), GrM-SA (1 μM) (open diamonds), Paclitaxel (Taxol) (5 μM) (closed triangles), or buffer alone (closed circles). Except for taxol, treated samples were pre-incubated for 2 h at 30°C Microtubule polymerization was then initiated by the addition of GTP (1 mM) and 5% glycerol and measured kinetically at 340 nm at 37°C Data represent the mean of 3-4 independent experiments. B, After 60 min of measurement, cleavage of bovine α-tubulin by GrM was verified by immunoblotting, using an anti-α-tubulin antibody.*

cleaves α-tubulin after other amino acids than the proposed Leu or Met [22,23]. The latter would be consistent with a Ser residue being a GrM cleavage site in ICAD [12]. Cleavage at alternate sites in substrates has also been found for GrA and its substrates Ku70 and SET [9]. Cleavage by GrM after Leu^{269} bisects the microtubule associated protein (MAP)-binding domain of α-tubulin (Fig. 6E) and this cleavage site is conserved in all human α-tubulin isoforms except α-tubulin-L3 (Fig. 6F). The latter isoform, however, harbors P1 Met that can also be hydrolyzed by GrM [22,23]. Thus, α-tubulin proteolysis by GrM is direct, efficient, complex, and occurs at multiple cleavage sites. One cleavage site includes Leu^{269} and at least one other cleavage site is positioned slightly more C-terminal thereof.

GrM de-regulates tubulin polymerization dynamics

To investigate the effect of GrM on tubulin polymerization rates, we pre-incubated GrM with a mixture of purified, MAP-depleted, bovine α- and β-tubulin. Following the addition of GTP, microtubule formation was measured kinetically at an absorbance of 340 nm. The kinetics of tubulin polymerization was markedly enhanced in the presence of GrM as compared with GrM-SA or buffer (Fig. 7A). This effect of GrM was comparable to the effect induced by the well established anti-microtubule anti-cancer drug paclitaxel (taxol) (Fig. 7A) [18,19]. GrM

cleaved bovine α-tubulin during the time course of tubulin polymerization (Fig. 7B). This is consistent with the GrM cleavage site (at least P4-P4') being completely conserved in bovine α-tubulin and multiple other mammalian species. Thus, GrM de-regulates the polymerization dynamics of the microtubule network.

GrM disorganizes the microtubule network during killing of tumor cells

To evaluate the physiological effects of GrM-mediated cleavage of α-tubulin, perforin was used to load Jurkat cells with GrM or GrM-SA for 4 h. GrM-induced cell dead was verified by PI flow cytometry (Fig. 3E-H) and visual morphological inspection (Fig. 3A-D) [10]. To visualize microtubules, cells were stained with an antibody against α-tubulin and analyzed by confocal microscopy (Fig. 8). The microtubule network of control perforin/GrM-SA treated cells appeared as a normal fine structured filamentous tubule network (Fig. 8A-B), which was similar to untreated cells and cells that were incubated with perforin alone (data not shown). In contrast, many cells that received perforin in combination with GrM displayed an aberrant microtubule network in that α-tubulin structures were less organized and appeared more diffuse (Fig. 8C-D). Interestingly, perforin/GrM-treated cells were more flattened and displayed an aberrant shape as compared to controls. The thickness of representative cells (mean +/- S.D.) was 6.6 (+/- 0.7) μm and 3.6 (+/- 0.3) μm ($P < 0.001$, $n = 7$) and the diameter was 12.3 (+/- 1.1) μm and 17.7 (+/- 1.4) μm ($P < 0.001$, $n = 7$) for GrM-SA- and GrM-treated cells, respectively. These data indicate that GrM disorganizes the microtubule network and cell shape in tumor cells that are attacked by perforin and GrM.

Discussion

Little is known about the molecular mechanisms by which GrM kills its target cells. It has been postulated that GrM uses multiple pathways to kill, either via caspase-dependent routes that lead to DNA fragmentation and ROS production, and via caspase-independent pathways that do not result in fragmentation of DNA and production of ROS [10-12]. Targeting of multiple independent cell death pathways has also been demonstrated for GrA and GrB [1,2,5-9]. In the present study, we have demonstrated that GrM neither cleaves nor activates pro-caspase-3 (Fig. 4), which is in contrast to Lu et al. [12], but consistent with Kelly et al. [10]. We have identified several novel potential substrates of GrM. We have shown that GrM directly cleaves the actin-plasma membrane linker ezrin and the microtubule network protein α-tubulin in tumor cells that are attacked by perforin and GrM (Figs. 3, 5, 6). Ezrin and α-tubulin are not cleaved by GrM in a caspase(-3)- or cysteine protease-dependent manner (Fig. 4). Cleavage of α-tubulin by GrM deregulates α-tubulin function and leads to disorganization of the microtubule network (Fig. 7, 8). Therefore, tubulin proteolysis by GrM is likely to be a critical event during NK cell-mediated killing.

Microtubules are responsible for cell survival, mitosis, motility, maintenance of cell shape, cell signaling, and intracellular trafficking of macromolecules, vesicles, and organelles [18,19]. The highly dynamic behavior of microtubules is greatly affected by well known anti-cancer drugs, like vinblastine, vincristine, and taxol, which all induce abnormal mitosis and cell death [18,19]. Furthermore, down regulation of α-tubulin by RNA interference results in death of tumor cells and limits their mitotic potential [15]. We have found that GrM cleaves off the C-terminal part of the α-tubulin MAP-binding domain (Fig. 6), which regulates

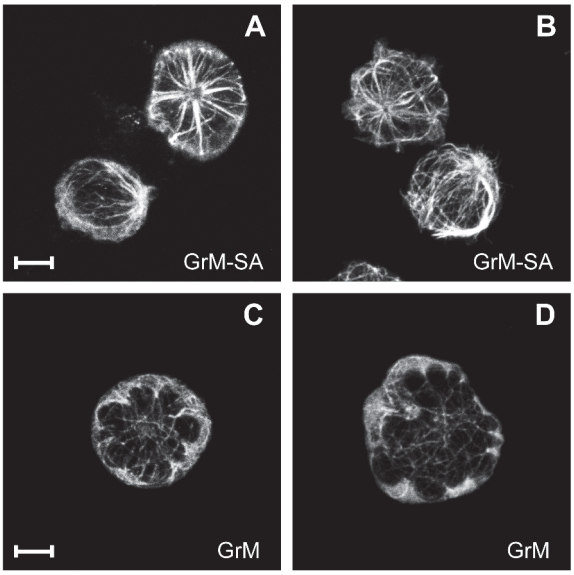

Figure 8. GrM disorganizes the microtubule network in tumor cells undergoing cell death. *A-D, Jurkat cells (1 x 10⁶) were treated with a sublytic dose of perforin (40 ng/ml) and GrM-SA (1 μM) (A, B) or GrM (1 μM) (C, D) for 4 h at 37°C α-Tubulin is visualized by X100 fluorescent immuno-staining and confocal microscopy. Bar, 5 μm.*

microtubule polymerization dynamics and microtubule motor activity [18,19]. Indeed, GrM, like taxol, shifted the balance of microtubule dynamics towards polymerization (Fig. 7), which has also been demonstrated for GrB [15]. In this context, however, we cannot fully exclude (i) that GrM affects in vitro tubulin polymerization by cleaving β-tubulin or trace amounts of MAPs that may accompany tubulins in the tubulin polymerization assay and/or (ii) that GrM-cleaved α-tubulin is irrelevant to microtubule polymerization in that it is more prone to aggregation as compared to uncleaved α-tubulin. Nevertheless, GrM disorganized the microtubule network and cell shape in tumor cells that are attacked by perforin and GrM (Fig. 8). Therefore, we hypothesize that GrM-induced cleavage of α-tubulin in tumor cells contributes to cell death induced by NK cells and/or that it enhances NK cell function to kill. The latter possibility would be compatible with the finding that taxol pre-treatment amplifies NK cell-mediated lysis of tumor targets [24]. Alternatively, it has been well established that host cell microtubules are indispensable for viral entry, replication, and exit [25]. GrM plays a significant role in the elimination of virus-infected cells in vivo [26]. This opens the possibility that GrM-mediated disruption of microtubule function terminates viral production in infected cells during NK cell attack. GrM may act in concert with GrB, since the latter also cleaves α-tubulin and resides in the same NK cell granules [15,20].

GrM cleaved the actin-plasma membrane linker ezrin, using purified proteins (Fig. 5), tumor cell lysates (Fig. 2), and tumor cells undergoing cell death induced by GrM and perforin (Fig. 3). Ezrin is (over)expressed in a variety of cancers, some of which are associated with poor clinical outcome [27]. Linkage of the plasma membrane to the actin cytoskeleton by ezrin allows a cell to interact directly with its microenvironment [27]. Ezrin also facilitates several

signal transduction pathways, like that of the protein kinases AKT and MAPK (MEK / ERK) that protect cells against apoptosis [16]. Therefore, GrM-dependent cleavage of ezrin may impair activation of AKT and MAPK survival pathways and thus may contribute to target cell death. Another possibility may be that GrM inhibits tumor metastatic progression by inactivation of ezrin. This would be consistent with the findings that ezrin is necessary for metastatic progression and that it is required for early metastatic survival of tumor cells in vivo [27]. Further studies are required to distinguish between these possibilities.

Fourteen novel potential substrates of GrM were identified (Table I). Apart from α-tubulin and ezrin, however, direct processing by GrM of these proteins and the precise role thereof remains to be investigated. Some potential GrM substrates may play a role in the mechanism by which GrM induces cell death, for instance proteins involved in chaperone and cell stress response (Table I). HSP90β plays an essential role in maintaining stability and activity of its client proteins, including a set of signaling proteins that regulate key pathways in cell survival and oncogenesis [28]. HSP90β is frequently over-expressed in cancer cells and more importantly synthetic HSP90β inhibitors have successfully been evaluated in multiple phase-II anticancer clinical trials [27]. If GrM indeed inactivates HSP90β, this may represent a novel mechanism by which GrM induces target cell death. We are currently addressing this possibility. Interestingly, the HSP90 co-chaperones Hop and Hip have recently been identified as novel substrates of GrB [29,30]. Whether or not GrM also plays a role in other cellular processes than cell death remains an intriguing question that deserves further study.

Our proteomic approach has identified a limited set of potential GrM substrates (Fig. 1, Table I). The relatively small number of cleavage events detected suggests that GrM substrate specificity depends on extended binding site(s) on folded proteins, rather than short linear peptides that represent the cleavage site (P1), i.e. methionine or leucine [22,23]. This is consistent with the high specificities of GrA and GrB, which also fully depend on secondary structures of folded substrates [31,32]. Remarkably, our proteomic screen did not detect one of the known GrM substrates, i.e. inhibitor of caspase-dependent DNase (ICAD), poly (ADP-ribose) polymerase (PARP), heat shock protein 75 (TRAP1), or pro-caspase-3 [11,12]. Missing of the latter is consistent with our finding that GrM neither cleaves nor activates pro-caspase-3 in tumor cells (Fig. 4). Although 2D-gel electrophoresis is capable of resolving more than thousand individual protein spots on a single gel, not all proteins could be visualized because of low abundance or extremes of molecular weight or charge. In addition, some of the known GrM substrates may have been detected on the gels, but could not be identified by tandem MS. We were able to definitively identify 16 of 37 excised protein spots. Because of the non-quantitative nature of silver staining, it remains difficult to address the cellular abundance of our identified potential GrM substrates.

GrM is highly expressed by NK cells, but not in CTLs [33]. NK cells play a major role in the innate immune response that forms the first line of defense against tumor cells and virus-infected cells, and they have broad applications in immunotherapy of cancer [34]. Knowledge of the precise mechanisms by which NK cells kill tumor cells may lead to further optimization of immunotherapy and/or other pro-apoptotic anticancer therapies.

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Chapter 6

The intracellular serine protease inhibitor SERPINB4 inhibits the proteolytic activity of human granzyme M

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Manuscript in preparation

Abstract

Granzyme (Gr)-mediated cell death is the major pathway for cytotoxic lymphocytes to kill virus-infected and tumor cells. In humans, five different granzymes (i.e. A, B, H, K, and M) are known that all induce cell death. Expression of intracellular serine protease inhibitors (serpins) is one of the mechanisms by which tumor cells evade cytotoxic lymphocyte-mediated killing. Intracellular expression of SERPINB9 by tumor cells renders them resistant to GrB-induced apoptosis. In contrast to GrB, however, no physiological intracellular inhibitors are known for the other four human granzymes. In the present study, we show that recombinant SERPINB4 (SCCA-2) formed a typical serpin-protease SDS-stable complex with both recombinant and native human GrM. Mutation of the P2-P1-P1' triplet in the SERPINB4 reactive centre loop completely abolished complex formation with GrM. N-terminal sequencing revealed that GrM cleaves SERPINB4 in its reactive centre loop after P1-Leu. Kinetic analysis with a chromogenic small synthetic GrM substrate demonstrated that SERPINB4 inhibited GrM activity with a stoichiometry of inhibition of 1.6 and an apparent second order rate constant of $2.6x10^2$ $M^{-1}s^{-1}$. In a tumor cell lysate, SERPINB4 inhibited cleavage of the macromolecular GrM-substrates α-tubulin and nucleophosmin. As SERPINB4 is highly expressed by squamous cell carcinomas, our current results suggest that SERPINB4 expression constitutes a novel mechanism by which these tumor cells evade cytotoxic lymphocyte-induced GrM-mediated cell death.

Abbreviations

CTL	Cytotoxic T lymphocyte
GFP	Green fluorescent protein
Gr	Granzyme
k_{inh}	Apparent second order rate constant
k_{obs}	First order rate constant
NK cell	Natural killer cell
RCL	Reactive centre loop
SCC	Squamous cell carcinoma
Serpin	Serine protease inhibitor
SI	Stoichiometry of inhibition

Introduction

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells (i.e. cytotoxic lymphocytes) play a pivotal role in the effector arm of the immune response that eliminate virus-infected cells and tumor cells [1]. Cytotoxic lymphocytes predominantly destroy their target cells by releasing the content of their cytolytic granules. These granules contain perforin and a family of unique structurally homologous serine proteases known as granzymes [2]. While perforin facilitates the entry of granzymes into the target cell, the latter induce cell death by cleaving critical intracellular substrates [3].

In humans, five different granzymes (GrA, GrB, GrH, GrK, and GrM) are known that differ on the basis of their substrate specificity [4]. All granzymes induce cell death with partially overlapping morphological hallmarks [4]. While GrA and GrB have been extensively studied, far less is known about the molecular cell death mechanisms of the other human granzymes. Recently, it has been demonstrated that GrM, which is highly expressed by NK cells, NKT cells, and γδ-T cells [5,6], mediates a major and novel perforin-dependent cell death pathway that plays a significant role in NK cell induced death [7]. In tumor cell lines, GrM directly and efficiently cleaves a diverse set of substrates, i.e. ICAD, PARP, HSP75, ezrin, α-tubulin, PAK 2, and nucleophosmin [8-11].

Tumor cells can escape from cytotoxic lymphocyte-induced killing by expression of cell death inhibitors in their cytoplasm, like the caspase-inhibitors XIAP and FLIP [12,13], and the GrB-inhibitor SERPINB9 (PI9) [14]. SERPINB9 is the only known intracellular human granzyme inhibitor and protects against GrB-induced apoptosis [14,15]. Moreover, SERPINB9 expression is associated with a poor clinical outcome in various types of tumors (e.g. lymphomas and melanomas) [16-18]. SERPINB9 belongs to the intracellular (B-clade) sub-family of human serine protease inhibitors (serpins), which share a unique inhibitory mechanism. Upon cleavage by a specific target protease in their reactive centre loop (RCL), serpins undergo a conformational change after which the serpin and the target protease are covalently bound, leaving the latter kinetically inactive [19].

In contrast to GrB, no physiological intracellular inhibitors are known for the other four human granzymes. As GrM is a very potent specialized inducer of cell death and displays a restricted cleavage site specificity in that it cleaves after P1-Leu and, to a lesser extent, after P1-Met [8,11,20,21], the aim of the current study was to identify an intracellular inhibitor of human GrM. Among the family members of intracellular serpins, SERPINB4 (squamous cell carcinoma antigen 2 (SCCA2) / leupin) is the only one that harbours a Leu at the putative P1-position in its RCL [22,23]. Here, we demonstrate that SERPINB4 binds to human GrM and inhibits its proteolytic activity towards a small synthetic peptide and natural macromolecular substrates. This might reflect a novel mechanism by which tumor cells evade GrM-mediated killing by cytotoxic lymphocytes.

Materials and methods

Recombinant proteins

Expression and purification of recombinant human GrM and the catalytically inactive GrM-SA variant was performed as described previously [8]. Briefly, cDNA encoding mature human GrM (residues Ile^{26}-Ala^{257}) was cloned into yeast expression vector pPIC9 (Invitrogen,

Paisley, UK). Catalytically inactive GrM-SA, in which the Ser^{195} residue in the catalytic centre is replaced by Ala, was generated by site-directed mutagenesis (Stratagene, Cedar Creek, TX). Plasmids were transformed into the GS115 strain of *P. pastoris* (Invitrogen) and granzymes were expressed in conditioned media for 72h. Recombinant GrM and GrM-SA were purified to homogeneity by cation-exchange chromatography (GE Healthcare, Diegem, Belgium) and dialyzed against 50 mM Tris (pH 7.4) and 150 mM NaCl. Recombinant GrM, but not GrM-SA, was active as determined by a synthetic chromogenic leucine substrate (Bachem, Weil am Rhein, Germany).

Preparation of recombinant SERPINB4 wild type (wt) and SERPINB4 RCL-mutant was performed using the expression vector pRSETC (Invitrogen) as described previously [24]. SERPINB4 wt, coding an N-terminal His_6-tagged fusion protein, and SERPINB4 RCL-mutant, in which the P2-Glu^{353}, P1-Leu^{354}, P1'-Ser^{355} amino acids were mutated into P2-Gln^{353}, P1-Gly^{354}, P1'-Ala^{355}, were expressed in. E.coli BL21 (DE3) using Overnight Express auto inducing medium (Merck, Nottingham, UK) containing 100 µg/ml ampicillin. Following growth at 37°C for 24h, cells were harvested by centrifugation at 15,000 g for 30 minutes and lysed using Bugbuster (Merck) lysis reagent. Soluble material was clarified by centrifugation of the lysate at 21,000 g for 30 minutes at 4°C. The recombinant serpin was purified using a His-Bind Purification kit (Qiagen, West Sussex, UK). Pooled imidazole eluted fractions were buffer exchanged into 50 mM Tris pH 8.0 and recombinant protein was stored at –80°C until required.

Generation of a novel mAb against human GrM

Mice were immunized with purified recombinant human GrM. Obtained hybridomas were screened for antibodies that reacted with GrM in different applications as previously described [25]. Anti-GrM mAb 3D4D7 (IgG1 isotype) was highly sensitive against immunoblotted recombinant GrM as well as native GrM from NK cell lysates (Figure 1). Cross-reactivity of mAb 3D4D7 against recombinant human GrB and GrK was excluded (results not shown).

Cell culture and transfection

293T and Jurkat cells were maintained in DMEM and RPMI-1640 medium (Invitrogen), respectively, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). The human NK cell line KHYG-1 [26] was purchased from the Health Science Research Resources Bank (JCRB0156) of the Japan Health Sciences Foundation and cultured similarly to Jurkat cells, with the addition of 50 ng/ml recombinant human interleukin 2 (Wako, Osaka, Japan). The plasmid pEGFP-N1 containing C-terminal green fluorescent protein (GFP)-tagged SERPINB4 (kindly provided by Dr. Wun-Shaing W. Chang, National Institute of Cancer Research, National Health Research Institutes, Taiwan, ROC) or mock control vector were transiently transfected into 293T cells using linear polyethylenimine (PEI) (Polysciences, Warrington, PA), according to the manufacturer's instructions. Cell lysates were prepared by three freezing-thawing cycles in liquid nitrogen of cells resuspended in a buffer containing 20 mM Tris (pH 7.4) and 150 mM NaCl. Protein concentrations of the supernatants were measured according to the procedure of Bradford (Bio-Rad, Hercules, CA).

Analysis of complex formation by SDS-PAGE and immunoblotting

Indicated amounts of purified recombinant SERPINB4 and GrM proteins were incubated

in 20 mM Tris (pH 7.4) and 150 mM NaCl for 1h at 37°C. When noted, Jurkat or KHYG-1 cell lysate was incubated with the recombinant protein(s) for the indicated times at 37°C. Samples containing recombinant proteins only were additionally treated with PNGase F (New England BioLabs, Ipswich, MA), according to the manufacturer's instructions. Subsequently, samples were separated on 10% SDS-PAGE gels under reducing conditions. For SDS-PAGE analysis, gels were stained with SimplyBlue (Invitrogen), according to the manufacturer's instructions. For immunoblot analysis, proteins were transferred onto immobilon–P membranes (Millipore, Billerica, MA). After blocking with 5% (w/v) Marvel dried skimmed milk (Premier International Foods, Coolock, UK) in TBS-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20), membranes were incubated for two hours at RT with our novel mouse anti-human GrM mAb (clone 3D4D7; 1.0 μg/ml), mouse anti-human α-tubulin mAb (clone B-5-1-2; 0.4 μg/ml) (Sigma), mouse anti-human nucleophosmin (clone FC-61991; 2.0 μg/ml) (Invitrogen), or mouse anti-human β-actin mAb (clone 2A2.1) (US Biological, Swampscott, MA). Goat anti-mouse IgG+IgM HRP (Biosource, Camarillo, CA) conjugate was used as secondary antibody. Bound antibodies were visualized using 3,3'-diaminobenzidine (DAB) (0.6 mg/ml) or enhanced chemiluminescence substrate (ECL) (GE Healthcare).

Immunoprecipitation

Cell lysates of mock and GFP-tagged SERPINB4 transfected 293T cells were incubated with 10 μg/ml anti-GFP mAb (Roche, Mannheim, Germany) and protein A/G plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA), and rotated end-over-end in non-sticky microfuge tubes (Ambion, Austin, TX) for 16h at 4°C. Precipitates were washed three times with 50 mM Tris (pH 7.4), 150 mM NaCl and incubated with 2.3 μM rh-GrM for 1h at 37°C. Next, the samples were washed twice more and finally boiled in reducing Leammli sample buffer. Immunoblotted samples were stained by Coomassie Brilliant Blue (BioRad) and the cleavage-product of interest was excised and identified by Edman degradation, using a 476A protein sequencer (Applied Biosystems, Foster City, CA).

Stoichiometry of inhibition

Increasing concentrations (0-3 μM) of recombinant SERPINB4, purified SERPINA1 (Calbiochem, Darmstadt, Germany), or SERPINA3 (Calbiochem) were incubated with a constant amount of recombinant human GrM (2 μM) in 50 mM Tris (pH 7.4) and 150 mM NaCl for 2h at 37°C. Samples were diluted 10-fold in 100 mM Tris (pH 7.4), 200 mM NaCl, and 0.01% Tween containing 1 mM synthetic chromogenic leucine substrate (AAPL-pNA) (Bachem) to terminate the reactions and transferred to a microplate (Greiner, Kremsmunster, Austria). The velocity of substrate hydrolysis by residual active GrM was measured at A_{405} using a microtiter plate reader (Anthos, Cambridge, UK). The fractional activity (velocity of GrM with a serpin / velocity of GrM only) was plotted against the ratio of $[serpin]_0$ / $[GrM]_0$. Linear regression analysis was used to determine the x-axis intercept as a value for the stoichiometry of inhibition.

Apparent second order constant

The interactions of SERPINB4, SERPINA1, and SERPINA3 with GrM were determined under pseudo-first order conditions by the progress curve method [27]. Recombinant GrM (0.2 μM) was added to 1 mM chromogenic substrate (AAPL-pNA) (Bachem) and different

concentrations of the serpins (0 – 3.2 µM). When indicated, 10 U/ml Heparin (Sigma) was included as well. The rate of substrate hydrolysis by GrM was measured in time at A_{405} using a microtiter plate reader (Anthos). Since serpin-serine protease interactions are irreversible, the time dependence of product accumulation on v_0 and k_{obs} is described in equation (1).

Equation (1): $[P] = v_0 / k_{obs} \times (1 - e^{-kobs \times t})$

Nonlinear regression analysis following equation (1) of each curve revealed the accessory first-order rate constants (k_{obs}). The reciprocal of these first-order rate constants ($1/k_{obs}$) were plotted against the reciprocal of the serpin concentrations (1/[serpin]). The apparent second order rate constant (k_{inh}) was calculated from the slope of this line: 1 / slope.

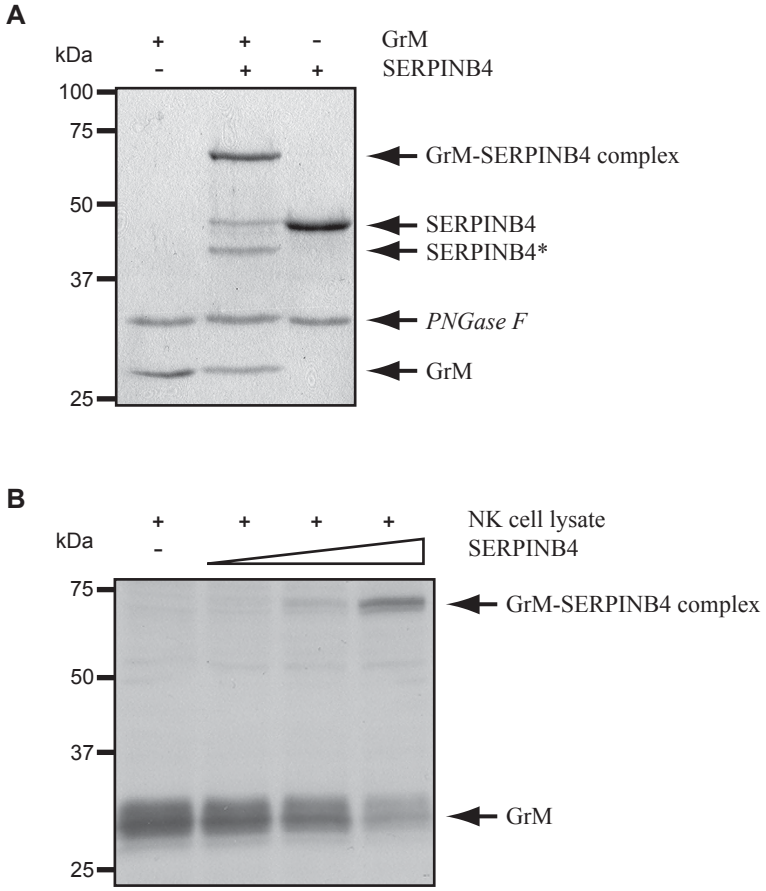

Figure 1. SERPINB4 forms a typical serpin-protease SDS-stable complex with recombinant and native human GrM. *A) Purified recombinant GrM (1.8 µM) and SERPINB4 (1.8 µM) were incubated for 1h at 37°C. All samples were treated with PNGase F, separated by SDS-PAGE, and analyzed by SimplyBlue staining. B) Cell lysate of the NK cell line KHYG-1 (33 µg) was incubated with recombinant SERPINB4 (0, 50, 150, and 450 ng) for 2h at 37°C. Subsequently, samples were immunoblotted for native GrM.*

Results

SERPINB4 forms a typical serpin-protease SDS-stable complex with human GrM

Upon cleavage by a specific target protease, most serpins form SDS-stable covalent-bound complexes with that protease, in which the latter one becomes inactive [19]. First, we investigated complex formation of SERPINB4 with human GrM by SDS-PAGE analysis using purified recombinant proteins (Figure 1A). Purified recombinant GrM and SERPINB4 migrated with a molecular mass of 28 and 44 kDa, respectively (Figure 1A, left and right lane). Incubation of GrM with SERPINB4 revealed an SDS-stable complex band with the expected molecular mass of around 70 kDa (Figure 1A, middle lane). The majority of SERPINB4 molecules formed a complex with GrM, whereas a small fraction of SERPINB4 was cleaved by GrM without trapping this protease. Second, we studied whether SERPINB4 could also bind to and form an SDS-stable complex with native human GrM (Figure 1B). The human NK cell line KHYG-1 was used as a source for native GrM. Incubation of a KHYG-1 cell lysate with recombinant SERPINB4 and subsequent immunoblotting for the protease revealed the formation of a complex between native GrM and recombinant SERPINB4 in a concentration-dependent way (Figure 1B). These data demonstrate that SERPINB4 forms a classical SDS-stable serpin-protease complex with both recombinant and native human GrM.

GrM cleaves SERPINB4 in its RCL after P1-Leu

To investigate if complex formation of SERPINB4 with GrM depends on the RCL and corresponding conformational change of the serpin, a RCL-mutant of SERPINB4 was included in which the amino acids at the putative P2(Glu)-P1(Leu)-P1'(Ser) positions were mutated. Western blot analysis for GrM revealed a complex with a molecular mass of about 70 kDa when the purified recombinant wild type (wt) proteins of GrM and SERPINB4 were incubated (Figure 2A). In contrast, no complex formation was detected upon incubation of SERPINB4 RCL-mutant with GrM-wt. Furthermore, a mutated catalytically inactive counterpart of GrM was included. As expected, this GrM-SA was not able to form a complex with SERPINB4-wt (Figure 2A). Thus, complex formation of SERPINB4 with GrM appears to depend on the RCL of the serpin.

Next, the exact cleavage site of GrM in the RCL of SERPINB4 was determined. Therefore, cell lysates of 293T cells expressing either C-terminal GFP-tagged full length SERPINB4 or an empty vector were incubated with GrM. Immunoblotting for GFP showed a C-terminal cleavage product of SERPINB4 (SERPINB4*-GFP) with the expected molecular mass of about 29 kDa (Figure 2B), as the RCL of SERPINB4 is near its C-terminus and the molecular mass of the linked GFP is about 25 kDa. Immunoprecipitation of GFP-conjugated SERPINB4 from 293T cell lysate and N-terminal sequencing of the subsequent GrM-induced C-terminal cleavage fragment revealed that GrM indeed cleaves SERPINB4 in its RCL after the amino acid Leu^{354} at the P1-position in the sequence ^{351}VVEL↓SSPS^{358}.

Stoichiometry of inhibition of GrM and SERPINB4

The interaction of a serpin with its physiological target protease usually results in complex formation with a stoichiometry of inhibition (SI) value close to 1. The SI value indicates how many serpin-molecules are needed to inhibited one molecule of target protease. If a serpin is also a substrate in parallel to an inhibitor of its target protease, this is reflected by a SI

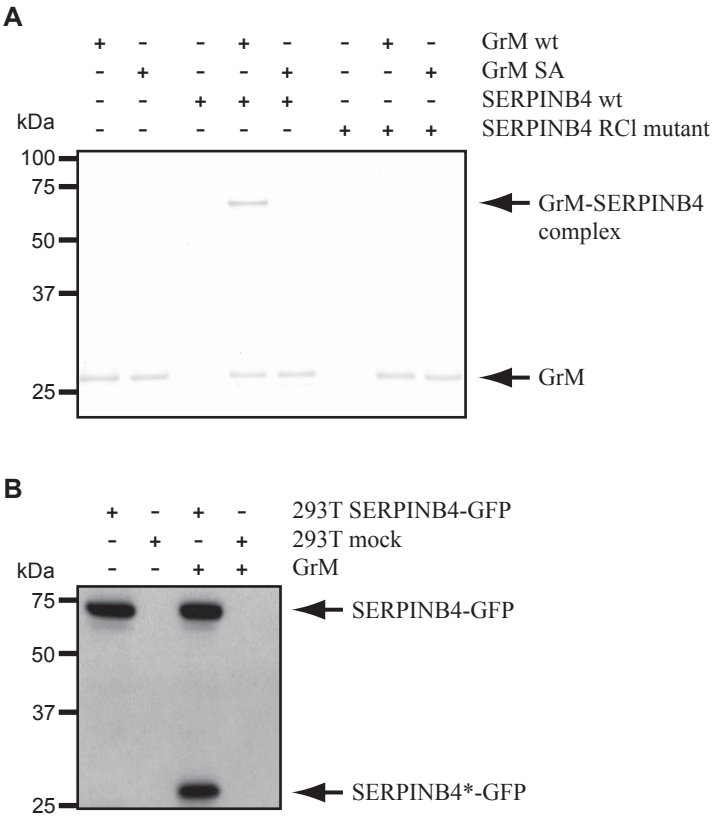

Figure 2. Mutation of the SERPINB4 RCL at the P2-P1-P1' positions completely abolish complex formation with human GrM. *A) Purified recombinant GrM (0.9 µM), GrM-SA (0.9 µM), SERPINB4 wt (0.9 µM), and SERPINB4 RCL-mutant (0.9 µM) were incubated for 1h at 37°C. All samples were treated with PNGase F, separated by SDS-PAGE, and immunoblotted for GrM. B) Cell lysates of 293T cells transfected with C-terminal GFP-conjugated SERPINB4 or an empty vector (mock) were incubated with recombinant GrM (0.5 µM) for 1h at 37°C. Subsequently, samples were immunoblotted for GFP. SERPINB4-GFP represents the full length protein, whereas SERPINB4*-GFP depicts the C-terminal cleavage product.*

value greater than 1. The SI value for inhibition of GrM by SERPINB4 was determined by measurement of residual GrM activity at increasing $[SERPINB4]_0$ / $[GrM]_0$ ratios (Figure 3A). Complete inhibition of GrM activity was obtained at a $[SERPINB4]_0$ / $[GrM]_0$ ratio of 1.6 ± 0.07 (mean ± SD of 4 independent experiments) (Figure 3A + Table 1). This indicates that about three molecules of SERPINB4 are needed to inhibit two GrM-molecules. In addition, the SI values of GrM and two extracellular serpins, SERPINA1 and SERPINA3, which are known to inhibit GrM activity [20], were determined. In agreement with Mahrus et al [20], these serpins inhibited human GrM with a SI of 1.1 and 1.6, respectively (Table 1), which is in the same range as SERPINB4. Thus, SERPINB4 forms an irreversible complex with human GrM and inhibits its activity with a SI value of 1.6.

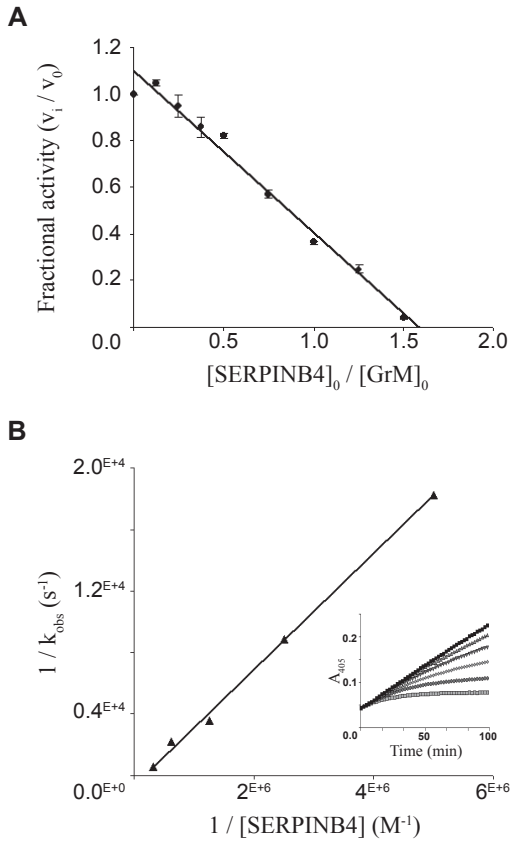

Figure 3. Kinetic analyses of GrM-inhibition by SERPINB4. *A) Purified recombinant human GrM (2 µM) was incubated with different concentrations of recombinant SERPINB4 (0 – 3 µM) for 2h at 37°C. Residual GrM activity was monitored by addition of a synthetic chromogenic leucine substrate (1 mM) and measuring A_{405} in time. The fractional activity (velocity of substrate hydrolysis by GrM in the presence of SERPINB4 / velocity of substrate hydrolysis by GrM without SERPINB4) was plotted against the ratio of $[SERPINB4]_0$ / $[GrM]_0$. Linear regression analysis was used to calculate the x-intercept as a value for the SI and determined to be 1.6 ± 0.07 (mean ± SD of 4 independent experiments). B) The progress curve method was used to determine the second order rate constant of the interaction between GrM and SERPINB4. Recombinant human GrM (0.2 µM) was added to a chromogenic leucine substrate (1 mM) and different concentrations of SERPINB4 (0 – 3.2 µM). The progress of GrM inhibition at different concentrations of SERPINB4 was measured in time at A_{405} (inset; ■: 0 µM, ▲: 0.2 uM, ▼: 0.4 uM, ♦: 0.8 uM, ●: 1.6 uM, □: 3.2 µM SERPINB4). Nonlinear regression analysis of each curve revealed the accessory first-order rate constants (k_{obs}). The reciprocals of these first-order rate constants ($1/k_{obs}$) were plotted against the reciprocals of the SERPINB4 concentrations (1/[SERPINB4]). The apparent second order rate constant (k_{inh}) was calculated from the slope of this line and determined to be 2.6 (± 0.1) x 10^2 $M^{-1}s^{-1}$ (graph represents 1 out of 3 independent experiments with similar results).*

Kinetic analysis of the inhibition of GrM activity by SERPINB4

To determine the rate of complex formation between GrM and SERPINB4, the apparent second order rate constant (k_{inh}) was measured under pseudo-first order conditions, using the progress curve method. GrM activity decreased in time upon the presence of SERPINB4 (Figure 3B, insert). Moreover, increasing concentrations of SERPINB4 resulted

Table 1. Kinetic constants of the interaction of human GrM with serpins.
SI: stoichiometry of inhibition
k_{inh}: apparent second order rate constant
n.d.: not determined

	SI	k_{inh} ($M^{-1}s^{-1}$)	k_{inh} + heparin
SERPINB4	1.6	2.6 x 10^2	2.5 x 10^2
SERPINA1	1.1	3.5 x 10^2	n.d.
SERPINA3	1.6	4.4 x 10^2	n.d.

in an enhanced decrease of GrM activity in time. Excessive concentrations of SERPINB4 over GrM completely inhibited GrM activity, again indicating the irreversibility of this complex formation. The progress of GrM inhibition at different SERPINB4 concentrations were represented as decays with a rate, k_{obs}, and plotted reciprocal against the SERPINB4 concentration (Figure 3B). The k_{inh} between SERPINB4 and GrM was calculated from the slope of this line and was determined to be 2.6 (± 0.1) x 10^2 $M^{-1}s^{-1}$, which is in the same range as both extracellular serpins that can inhibit GrM activity (Table 1). Because the k_{inh} of SERPINB4 and GrM is in the low range of serpin-kinetics, the glycosaminoglycan heparin was included in these experiments, which is a known co-factor for several serpins that enhances the k_{inh} towards their target proteases [28]. However, the k_{inh} for SERPINB4 and GrM was not increased by heparin (Table 1).

SERPINB4 inhibits cleavage of natural macromolecular substrates by GrM

The inhibitory effect of SERPINB4 on the activity of human GrM was further investigated using natural macromolecular substrates of GrM. After pre-incubation of recombinant GrM and SERPINB4 at various concentration-ratios, the residual GrM-activity towards two known direct GrM-substrates [8,11] in a Jurkat tumor cell lysate was determined by immunoblotting (Figure 4). As expected, in the absence of SERPINB4, human recombinant GrM efficiently cleaved the 55 kDa α-tubulin subunit as well as the 37 kDa nucleolar phosphoprotein nucleophosmin. Recombinant SERPINB4 impaired cleavage of both α-tubulin and nucleophosmin by GrM in a concentration-dependent manner (Figure 4). Excessive concentrations of SERPINB4 over GrM completely inhibited GrM activity towards α-tubulin, whereas some nucleophosmin cleavage was still observed, suggesting that nucleophosmin is a more efficient GrM-substrate than α-tubulin. β-actin served as a loading control (Figure 4). In conclusion, SERPINB4 inhibits GrM-mediated cleavage of the natural macromolecular substrates α-tubulin and nucleophosmin in a tumor cell lysate.

Discussion

The granule-exocytosis pathway is the major mechanism for cytotoxic lymphocytes to kill tumor and virus-infected cells. Tumor cells can evade GrB-mediated cell death by expression of the intracellular serine protease inhibitor SERPINB9. However, such physiological intracellular inhibitors are unknown for the other four human granzymes. In mice, GrM is

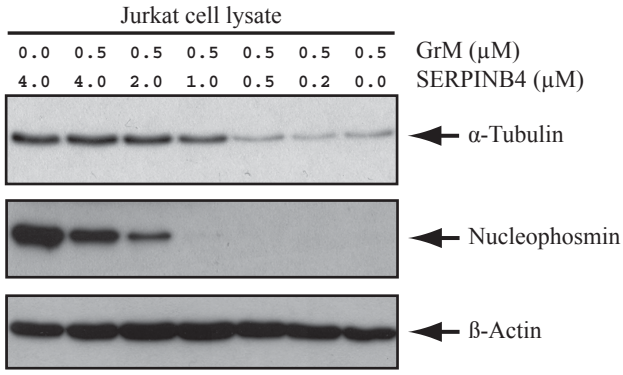

Figure 4. SERPINB4 inhibits GrM-mediated cleavage of macromolecular substrates. *Indicated concentrations of recombinant GrM and SERPINB4 were co-incubated for 2h at 37°C. Subsequently, Jurkat tumor cell lysate (2 μg) was added to the samples and incubated for another 4h at 37°C to determine the residual GrM-activity towards macromolecular substrates. Finally, all samples were immunoblotted for α-tubulin, nucleophosmin, and β-actin.*

inhibited by the murine serpin SPI-CI, which protects cells from perforin/GrM-mediated cell death [29]. SPI-CI is unique for mice and no human orthologue is known. In the present study, we show for the first time that human GrM also has an endogenous intracellular inhibitor that might protect cells from GrM-mediated killing. We have demonstrated that GrM cleaves SERPINB4 in its RCL after P1-Leu, resulting in the formation of a typical serpin-protease SDS-stable complex (Figure 1-2). Furthermore, SERPINB4 inhibited the proteolytic activity of GrM towards natural macromolecular substrates (Figure 4).

Our findings are consistent with the facts that GrM preferably cleaves after a P1-Leu and, to a lesser extent, after P1-Met [20,21], and that SERPINB4 harbours a Leu at the P1-position in its RCL [22,30]. Although SERPINB4 is the only intracellular serpin that harbours a Leu or Met amino acid at the putative P1-position in its RCL [23,31], we cannot fully exclude that other intracellular serpins than SERPINB4 inhibit human GrM. Currently, five out of thirteen B-clade serpin-members (i.e. SERPINB1, -B4, -B6, -B8, and -B9) are each known to use more than one RCL P1-residue to inhibit different target proteases, thereby broadening the inhibitory profile of a single serpin [31]. Interestingly, the GrB-inhibitor SERPINB9 harbours a Met residue at the putative P3' position in its RCL. Indeed, GrM cleaves SERPINB9, however, an SI value of 59 points to a substrate rather than an inhibitor [20]. Therefore, it has been proposed that cleavage and inactivation of SERPINB9 by GrM clears the way for GrB-induced target cell killing [20]. Whether other intracellular serpins than SERPINB4 exist that inhibit human GrM activity remains an open question.

We showed that SERPINB4 forms a typical serpin-protease SDS-stable complex with both recombinant and native human GrM (Figure 1). Kinetic analysis of GrM-inhibition by SERPINB4 revealed a SI of 1.6 ± 0.07 (Figure 3A), indicating that about two-third of the SERPINB4 molecules forms a covalent complex with GrM and inhibits its activity, whereas about one-third of the SERPINB4 molecules is a substrate of GrM without trapping the protease. This corresponds with the relative intensity of the bands in Figure 1A representing complexed and cleaved SERPINB4. The SI of GrM with the extracellular human serpins SERPINA1 (SI = 1.1) and SERPINA3 (SI = 1.6) (Table 1) were similar as previously described [20], and in the same range of GrM and SERPINB4. Although a reasonable SI-value

was determined for SERPINB4 and GrM, the k_{inh} of 2.6 (± 0.1) x 10^2 $M^{-1}s^{-1}$ was into the low range as compared with other serpin-protease interactions. Previously, SERPINB4 revealed k_{inh}-values of 1.0 x 10^5 $M^{-1}s^{-1}$ and 2.8 x 10^4 $M^{-1}s^{-1}$ with cathepsin G and chymase, respectively [32]. Our kinetic analysis could therefore preclude a physiological role of the GrM-SERPINB4 interaction in a biological system. However, the k_{inh}-value for SERPINB4 and GrM might be affected by posttranscriptional modification of our recombinant human GrM preparation. For recombinant GrM expression, we have employed yeast *P. pastoris* that is known to (hyper)glycosylate secreted proteins. Because human GrM has three potential N-linked glycosylation motifs [33], our purified recombinant GrM batches contained both glycosylated and hyperglycosylated GrM variants (results not shown), as previously described by others [20]. Bulky carbohydrate groups on the surface of the GrM protein could negatively influence the affinity for binding to and/or inhibition by SERPINB4. For two extracellular serpin family members, SERPINA1 and SERPINA3, we (Table 1) and Mahrus et al [20] also demonstrate k_{inh}-values in the 10^2 $M^{-1}s^{-1}$ range using (hyper)glycosylated recombinant GrM protein, despite SDS-stable protein complexes and significant SI-values. Isolated native human GrM from lytic granules of cytotoxic lymphocytes is required to address this issue. In this context, it should be mentioned that the local concentration of SERPINB4 in e.g. tumor cells finally determines its functionality as physiological GrM-inhibitor.

In normal tissue, SERPINB4 is mainly expressed by stratified squamous epithelium of both the upper gastrointestinal tract and the female genitourinary system, and by pseudo-stratified columnar epithelium of the conducting airways [34]. Interestingly, tumors originating from these epithelial tissues also express SERPINB4, i.e. squamous cell carcinomas (SCC) of the cervix [35], head and neck, and lung [34]. Although SERPINB4 is a family member of the intracellular serpins and normally localizes exclusively in the cytoplasm of epithelial cells, it is often detected in serum of patients with a SCC [36,37]. Because SERPINB4 is not actively secreted by squamous carcinoma cells, it has been suggested that it is passively released into the circulation, likely after necrosis of tumor cells [38]. There, SERPINB4 serves as a serological marker for the diagnosis of SCC. Elevated circulating SERPINB4 levels in SCC-patients are associated with advanced stage of tumor progression and poor disease-free survival [39,40]. This indicates that expression of SERPINB4 is beneficial for tumor cells. Indeed, SERPINB4 inhibits both radiation- and TNF-induced apoptosis in transfected cell lines, probably by inhibition of the p38 MAPK pathway and the proteolytic activity of endogenous cathepsin G, respectively [24,41]. Our current findings that SERPINB4 binds to and inhibits the proteolytic activity of human GrM, suggest a novel function for SERPINB4, i.e. enhancement of tumor progression through interference with the granule-exocytosis cell death pathway of cytotoxic lymphocytes. This intriguing concept has been well studied for SERPINB9-expressing tumor cells that efficiently inhibit GrB-induced cell death [14]. SERPINB9 expression in several types of tumors is associated with a poor clinical outcome of patients [16,17]. In analogy with SERPINB9, SERPINB4 expression may constitute a novel mechanism by which squamous cell carcinoma tumor cells evade GrM-mediated cytotoxic lymphocyte-induced cell death.

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Chapter 7

Down-regulation of SERPINB13 expression in head and neck squamous cell carcinomas associates with poor clinical outcome

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Abstract

Tumorigenesis of head and neck squamous cell carcinomas (HNSCC) is associated with various genetic changes such as loss of heterozygosity (LOH) on human chromosome 18q21. This chromosomal region maps a gene cluster coding for a family of intracellular serine protease inhibitors (serpins), including SERPINB13. As SERPINB13 expression in HNSCC has recently been shown to be down-regulated both at the mRNA and protein levels, here we investigated if such a low SERPINB13 expression is associated with histopathological and clinical parameters of HNSCC tumors and patient survival. By generating specific antibodies followed by immunohistochemistry on a well-defined cohort of 99 HNSCC of the oral cavity and oropharynx, SERPINB13 expression was found to be partially or totally down-regulated in 75% of the HNSCC as compared to endogenous expression in non-neoplastic epithelial cells. Down-regulation of SERPINB13 protein expression in HNSCC was significantly associated with the presence of LOH at the SERPINB13 gene in the tumors (p=0.006), a poor differentiation grade of the tumors (p=0.001), the presence of a lymph node metastasis (p=0.012), and a decreased disease-free (p=0.033) as well as overall (p=0.018) survival of the patients. This is the first report demonstrating that down-regulation of SERPINB13 protein expression in HNSCC is positively associated with poor clinical outcome. Therefore, SERPINB13 seems to act as an important protease inhibitor involved in the progression of HNSCC.

Statements about novelty and impact

A disbalance between the proteolytic activity of a protease and the expression of its cognate inhibitor can lead to tumor progression. In the present study, we demonstrate for the first time that down-regulation of SERPINB13 protein expression in HNSCC is positively associated with metastasis of these tumors and poor clinical outcome of the patients.
Both the protease inhibitor SERPINB13 and its physiological target protease may act as important proteins involved in the progression of HNSCC. Identification of the physiological target protease of SERPINB13 and subsequent development of specific small synthetic inhibitors against this enzyme may lead to a novel therapeutic strategy to treat HNSCC.

Abbreviations

ELISA	Enzyme-linked immuno sorbent assay
GFP	Green fluorescent protein
Ig	Immunoglobulin
IHC	Immunohistochemistry
HNSCC	Head and neck squamous cell carcinomas
HR	Hazard ratio
LOH	Loss of heterozygosity
LN	Lymph node
mAb	Monoclonal antibody
Serpin	Serine protease inhibitor

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common tumor type worldwide with tobacco smoking and alcohol consumption as major risk factors [1]. Despite advances in surgery, radiation, and chemotherapy the prognosis for patients with HNSCC remains poor with a 5-year survival rate of approximately 50%, depending on stage. Although higher stage is associated with a poor performance, the prediction of the aggressiveness of these tumors based on clinical parameters alone is still difficult [2,3]. Identification of additional biological markers that help to predict aggressive tumor behavior can improve staging and may be used as novel diagnostic tools or even as targets for therapeutic intervention.

Previous studies have shown that tumorigenesis of HNSCC is correlated with various genetic changes such as loss of heterozygosity (LOH) of chromosome 18q21 [4,5]. A prognostic association between LOH of 18q and poor patient survival was also identified, indicating that loss of a tumor suppressor gene or genes in that chromosomal region may contribute to aggressive tumor behavior [6,7]. Chromosome 18q21 maps a cluster of genes coding for a family of proteins known as the intracellular serine protease inhibitors (serpins) [8-10], whose aberrant expressions are often associated with the malignant progression of HNSCC. For example, progression and invasion of HNSCC is marked by loss of SERPINB2 (PAI-2) expression [11], whereas over-expression of SERPINB3 (SCCA-1) in a HNSCC cell line inhibited tumor growth and invasion *in vivo* [12]. High expression of SERPINB5 (Maspin) was associated with absence of LN metastasis and improved survival of patients with oral SCC [13].

SERPINB13 (Hurpin, Headpin, PI13) is another family-member that maps to the serpin cluster at chromosome 18q21 [14]. Northern blot analysis of multiple normal tissues revealed that SERPINB13 is only expressed in normal oropharyngeal mucosa and skin [14,15]. Protein expression was confirmed for these two tissue types but other normal tissues were not investigated [16-19]. In HNSCC, SERPINB13 expression is down-regulated both at the mRNA and protein level [14,16,17]. However, the association of SERPINB13 expression with clinical outcome in patients with HNSCC has not yet been investigated.

The aim of the present study was to investigate if down-regulation of SERPINB13 protein expression in HNSCC is clinically relevant and associated with a more aggressive tumor behavior. Therefore, we generated novel specific monoclonal antibodies (mAbs) against SERPINB13 and examined its expression in a well-defined cohort of 99 SCC of the oral cavity and oropharynx, and their corresponding lymph node metastasis. We conclude that down-regulation of SERPINB13 protein expression in HNSCC is positively associated with poor clinical outcome.

Material and Methods

Expression and purification of recombinant SERPINB13 in *E.coli*

The human SERPINB13 cDNA was cloned after a His-tag into the bacterial expression vector pQE-80L (Qiagen Benelux, Venlo, The Netherlands). The *E.coli* BL21 codon-plus RIL strain (Stratagene, La Jolla, CA) was transformed with this plasmid according to the manufacturer's instructions and SERPINB13 expression was induced by isopropyl-β-d-thio-galactopyranoside (IPTG). Cells were harvested and lysed in bacterial protein extraction reagent (B-PER) (Pierce, Rockford, IL) followed by purification of recombinant SERPINB13

to homogeneity by metalchelate-affinity chromatography using Talon metal affinity resins (Clontech, Palo Alto, CA) according to the manufacturer's instructions and dialyzed to PBS.

Generation and selection of mAbs against SERPINB13

Immunization of mice, hybridoma culture, and selection of SERPINB13 mAbs were performed as previously described [20]. Briefly, mice were successively immunized with purified recombinant SERPINB13 from *E.coli*. The obtained hybridomas (n=768) were screened for affinity against SERPINB13 using ELISA, coated with recombinant SERPINB13. The positive clones (n=44) were screened in a second round by ELISA, immunoblotting, and immunohistochemistry (IHC), followed by limiting dilution. Finally, four clones were selected for their reactivity with SERPINB13 on Western blot and for their use in IHC on paraffin embedded tissues, similarly as previously described for other anti-serpin antibodies [21,22]. The mAb that performed best in IHC is referred to as 4A9D3, determined to be of the IgG1-isotype, and used in this study.

Cell culture and transfection

The human HaCaT keratinocyte cell line [23] and embryonic 293T kidney cell line were respectively cultured in RPMI-1640 and DMEM (Invitrogen, Paisley, UK) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma, St. Louis, MO), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Differentiation of HaCaT cells was induced by the addition of $CaCl_2$ (final Ca^{2+} concentration 1.4 mM) to the medium of confluent grown cells for 72 h. 293T cells were transiently transfected with the pEGFP-N1 plasmid containing C-terminal GFP-tagged SERPINB13 wild type or its two known splice variants [24]. Other members of the serpin B-clade sub-family such as SERPINB1, -B3, -B4, B5-, and -B10 were also expressed in 293T using the pEGFP-N1 vector, whereas the pBabe vector contained the cDNAs encoding for SERPINB6, -B8, and -B9. As control, empty vectors were included. Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Immunoblotting

Cell lysates were prepared by three freezing-thawing cycles in liquid nitrogen of cells resuspended in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl. Protein concentration of the supernatant was measured according to the procedure of Bradford (Bio-Rad, München, Germany). Twenty µg of protein was separated by electrophoresis on a 10% (w/v) SDS-PAGE under reducing conditions and subsequently transferred onto an immobilon-P membrane (Millipore, Billerica, MA). After blocking with 5% (w/v) Marvel dried skimmed milk (Premier International Foods, Coolock, UK) in TBS-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20), membranes were incubated for 2 h at RT with anti-SERPINB13 mAb 4A9D3 or anti-GFP mAb (Roche, Indianapolis, IN) in TBS-T containing 2.5% (w/v) Marvel, at a final concentration of 0.9 µg/ml and 0.4 µg/ml, respectively. Goat anti-mouse IgG+IgM HRP conjugate (Biosource, Camarillo, CA) was used as secondary antibody at a final concentration of 0.1 µg/ml and bound antibodies were visualized with a chemiluminescence development reagent (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions.

Normal tissues and cohort of HNSCC tumors

Formalin-fixed paraffin-embedded sections from all normal tissues, the panel of HNSCC, and their corresponding lymph node metastasis were obtained from the tissue bank of the Department of Pathology / UMCU Biobank, UMC Utrecht. Tissues were used in line with the code "Proper Secondary Use of Human Tissue" as installed by the Dutch Federation of Biomedical Scientific Societies. Normal tissues of the following locations were included: tongue, mouth, pharynx, nose, parotid and submandibular gland, vocal cord, trachea, lung (bronchus and peripheral), pleura, esophagus, stomach, small intestine, colon, peritoneum, liver, gallbladder, pancreas, testis/epididymis, prostate, bladder, kidney, urethra, ovarian, tuba, uterus, cervix, placenta, mamma, cerebrum, cerebellum, brain stem, meninges, spinal cord, nerve fiber, skin, thyroid, parathyroid, adrenal gland, pituitary gland, tonsil, lymph node, spleen, thymus, bone marrow, heart, epi/pericardium, skeletal muscle, blood vessels, eye, bone, cartilage, synovial joint, adipose tissue, and umbilical cord.

The cohort of primary HNSCC was previously studied [25] and consisted of 99 patients that were treated in our hospital between 1996 and 2001 for a primary SCC of the oral cavity or the oropharynx. None of these patients had a previous malignancy in the head and neck region. Representative formalin-fixed, paraffin-embedded tissue blocks of these 99 primary tumors were selected that also contained normal epithelium, serving as an internal control for endogenous SERPINB13 expression. In addition, representative tissue blocks from the largest tumor-positive lymph node (n=56) were selected as well. All H&E slides from these cases were re-reviewed by an experienced head and neck pathologist (JAK) for the following pathological features: tumor type, differentiation grade, diameter, infiltration depth, dysplasia, spidery growth, perineural-, bone-, and vaso-invasive growth, the presence of lymph node metastasis, and the diameter of the largest metastatic node. For each patient, the following clinical characteristics were noted from the medical records from the Departments of Oral and Maxillofacial Surgery and Otolaryngology, UMC Utrecht: age at diagnosis, sex, cTNM and pTNM, treatment modality, the occurrence of relapses, disease free survival time, overall survival time, and cause of death. Patient and tumor characteristics are shown in Table 1.

Immunohistochemistry

Four μm thick slides were cut and mounted on silane-coated glass slides. After deparaffinization and blocking endogenous peroxidase activity, the tissue sections were subjected to antigen retrieval by boiling in 10 mM sodium-citrate buffer, pH 6.0, for 10 min in a microwave. Subsequently, the tissue slides were washed with PBS, 0.05% (v/v) Tween-20. The anti-SERPINB13 primary antibody 4A9D3 was diluted in PBS, 1% (w/v) BSA to a final concentration of 4.5 μg/ml, and incubated for 1 h. Subsequently, the tissue slides were incubated with 1:250 diluted biotin-conjugated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA), followed by 1:400 diluted peroxidase-labeled streptavidin (Immunotech, Marseille, France). After washing with PBS, SERPINB13 was visualized using 3,3'-diaminobenzidine (0.6 mg/ml). Slides were counterstained with haematoxylin. Appropriate positive and negative controls were used throughout. Intensity and percentages of positive tumor cells were evaluated semi quantitatively and independently by 2 observers. In case of disagreement, the observers reanalyzed the staining results until they reached consensus. SERPINB13 expression was scored for both its intensity in tumor cells relative to normal epithelium (normal = 2, weaker = 1, total loss = 0) and the percentage of tumor cells in the tissue section with such a specific intensity. Multiplying of these two scoring variables

Table 1. Clinical and histopathological parameters of the cohort of HNSCC.

Characteristic	**No.**		
Patients	**99**		
Sex:		*Age at initial diagnosis (yrs)*	
Male	59	Median	61.4
Female	40	Range	38-87
HNSCC	**99**		
Location & sub-location:		*Perineural growth:*	
Oral cavity	86	Absent	55
Alveolus & gingiva	9	Present	41
Buccal mucosa	9		
Floor of mouth	34	*Spidery growth:*	
Tongue	34	Absent	20
Oropharynx	13	Present	77
Anterior wall	4		
Lateral wall	8	*Local recurrence (< 2 yrs):*	
Superior wall	1	No	60
		Yes	15
Infiltration depth (mm):			
≤ 10.0	43	*Distant metastasis (< 5 yrs):*	
> 10.0	56	No	36
		Yes	8
Differentiation grade:			
Good / Moderate	83	*Bone-invasion:*	
Poor / Undifferentiated	15	Absent	23
		Present	27
Vaso-invasion:			
Absent	69	*N-stage:*	
Present	24	pN0	43
		pN+	56
T-stage:			
pT1	17	*Radiotherapy:*	
pT2	34	No	29
pT3	15	Yes	70
pT4	33		

resulted in a scoring range of 0 till 200, in which a score of 0 represents a complete loss of SERPINB13 expression in all tumor cells and a score of 200 represents normal SERPINB13 expression throughout the tumor.

Loss of heterozygosity

LOH analysis of the SERPINB13 gene on chromosome 18q21.3 was performed successfully on 51 HNSCC. DNA was isolated from peripheral blood lymphocytes and corresponding HNSCC frozen tissue sections, of which the latter one consisted of at least 50% tumor cells. We used the microsatellite markers D18S51 and D18S814 for

the centromeric and telomeric side of the SERPINB13 gene, respectively. In detail, D18S51 5' primer: GAGCCATGTTCATGCCACTG (FAM-labeled), D18S51 3' primer: CAAACCCGACTACCAGCAAC, D18S814 5' primer: CTTCCCTGGGTATCAA-GACT (FAM-labeled), and D18S51 3' primer: TCCCACTATATGTATGTTCACC (Eurogentec, Seraing, Belgium). The PCR-mix contained 2 µl 10x buffer II, 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.5U AmpliTaq DNA polymerase (Roche, Mannheim, Germany), 2 pmol forward primer, 2 pmol reverse primer, and 50 ng DNA as input. Target DNA sequences were amplified for 10 cycles of 94°C for 15 s, 55 °C for 15 s, and 72°C for 30 s, followed by 20 cycles of 89°C for 15 s, 55°C for 15 s, and 72°C for 30 s. Amplification was preceded by a denaturing step at 94°C for 5 min and completed with an extension step at 72°C for 5 min. PCR products were detected on an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA), using ROX500 as standard marker. Data were analyzed using GeneScan Analysis and Genotyper software and interpreted as described by Ramal et al. [26]. Briefly, LOH was assigned when more than 25% signal reduction of one allele was observed in the tumor sample as compared to the control peripheral blood lymphocytes sample.

Statistical analysis of pathological and clinical data

The Mann-Whitney U test was used to analyze associations between SERPINB13 expression and the histopathological and clinical parameters. *p* values were based on two-tailed statistical analysis, in which $p<0.05$ was considered significant. Survival analysis was performed with the Kaplan-Meier and Cox regression method. Differences between survival curves were analyzed using the log-rank test for trend. All analyses were done using the SPSS statistical software (version 15.0) (SPSS, Chicago, IL).

Results

Characterization of a novel mAb against SERPINB13.

We have generated a panel of mAbs against SERPINB13 and selected mAb 4A9D3 for this study. This mAb revealed a specific band with immunoblotting of 293T cells expressing GFP-tagged wild type SERPINB13 as well as with the two known SERPINB13 splice-variants (Figure 1A, upper panel). These bands were detected at the expected molecular weight of ~71 kDa, as SERPINB13 wild type itself is 44 kDa and the GFP-tag adds an additional 27 kDa. mAb 4A9D3 showed no reactivity towards 293T cells transfected with a control empty plasmid and no cross-reactivity against any of the four most homologous, GFP-tagged intracellular serpins SERPINB1 (46% amino acid homology), -B3 (58%), -B4 (58%), and -B10 (44%) (Figure 1A upper panel). Expression of all GFP-tagged serpins was demonstrated by an anti-GFP mAb (Figure 1A, lower panel). Cross reactivity of mAb 4A9D3 with less homologous intracellular serpins, SERPINB5, -B6, -B8, and -B9, was also excluded (results not shown). Previously, others have reported that SERPINB13 is endogenously expressed in the human keratinocyte cell line HaCaT [15]. Indeed, our mAb revealed a band at 44 kDa in HaCaT cells (Figure 1B), indicating that it recognizes endogenous SERPINB13 as well. This band was even more abundant in differentiated HaCaTs (Figure 1B), which is consistent with the observation that SERPINB13 expression is up-regulated after differentiation of keratinocytes [15]. Taken together, our mAb 4A9D3 specifically recognizes recombinant and native SERPINB13.

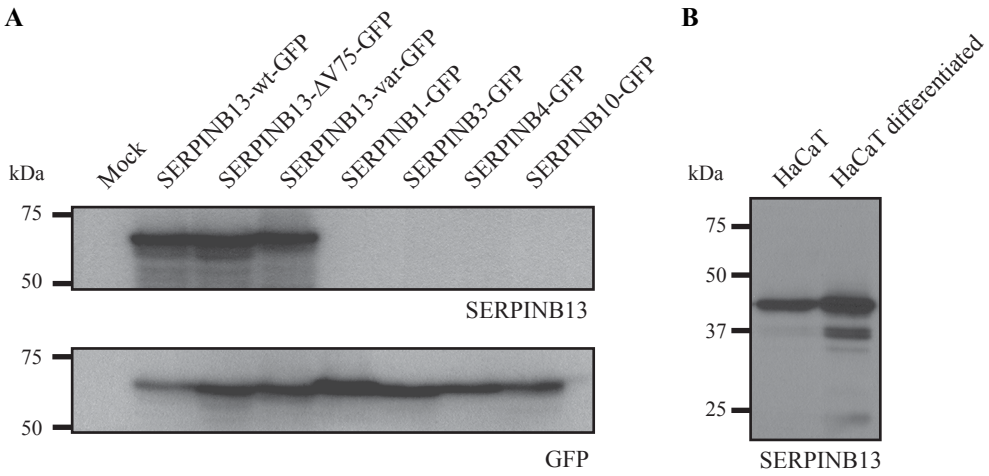


Figure 1. Characterization of a novel mAb (4A9D3) against human SERPINB13. *A) Western blot analysis of 293T cells transfected with GFP-tagged SERPINB13 wt, two SERPINB13 splice variants, and four other highly homologous intracellular human serpins. Upper panel: anti-SERPINB13 mAb 4A9D3. Lower panel: anti-GFP mAb. B) Western blot analysis of SERPINB13 expression in normal cultured and differentiated HaCaT cells using anti-SERPINB13 mAb 4A9D3.*

SERPINB13 is highly expressed in, but not fully restricted to, squamous epithelium.

The protein expression level of SERPINB13 in normal tissues was investigated by IHC against a large panel of human tissues listed in the materials and methods section. As described previously [16-19], we observed a strong SERPINB13 expression in keratinizing epithelium of the skin as well as in non-keratinizing mucosa covering various upper aero-digestive tract sites (Figure 2A-E). SERPINB13 was also expressed in squamous epithelium of the ectocervix, respiratory epithelium of the trachea and bronchus, and transitional epithelium of the bladder (Figure 2F-H). The expression levels in the latter two were weaker compared to that seen in the squamous epithelia. Interestingly, a significant expression of SERPINB13 was also identified in a sub-population of cells in the pituitary gland (Figure 2I). In all these tissues, the sub-cellular localization of SERPINB13 was both cytoplasmic and nuclear. No SERPINB13 expression was detected in other tissues or cell types. In conclusion, SERPINB13 is expressed primarily in keratinizing and non-keratinizing squamous epithelium, in respiratory and transitional epithelium, and in a subset of neuroendocrine cells of the pituitary gland.

SERPINB13 is down-regulated in HNSCC.

SERPINB13 protein expression was investigated in a panel of 99 primary HNSCC and 56 corresponding LN metastasis by IHC with scoring performed as aforementioned. Primary HNSCC were divided into three groups based on their SERPINB13 expression. Thirty-one tumors showed (almost) total loss of SERPINB13 throughout the tumor (score 0-50) (Figure 3A, upper panels). Forty-three tumors demonstrated partial loss of SERPINB13 protein expression (score 51-149) as a result of a weaker intensity of the expression and/or a decreased percentage of SERPINB13-expressing cells (Figure 3A, middle panels). No to marginal loss of SERPINB13 expression throughout the tumor was detected in 25 cases (score 150-200)

(Figure 3A, lower panels). Hence, SERPINB13 expression was down-regulated in 75% of the HNSCC patients (Figure 3B, left box). No difference of SERPINB13 expression was observed between primary HNSCC and their corresponding LN metastasis (mean score 92.7 and 92.2, respectively) (Figure 3B). These results demonstrate a clear partial or total down-regulation of SERPINB13 protein expression in the majority of both primary HNSCC and their corresponding LN metastasis.

In SERPINB13-positive tumors the staining was always cytoplasmic as well as nuclear, and the intensity of the nuclear staining was related with the cytoplasmic staining. SERPINB13 staining in tumor cells lining the outer rim of the tumor fields was nearly always weak or negative, even in SERPINB13-positive tumors. In tumors that showed only focal positivity, the staining was often still present in the areas of tumor with evidence of squamous differentiation in the form of extracellular keratin (keratin whorls).

Down-regulation of SERPINB13 protein expression is associated with the presence of LOH at the serpin cluster on chromosome 18q21.3 in HNSCC.

To investigate a possible underlying mechanism of the down-regulated SERPINB13 protein expression in our panel of HNSCC, we performed LOH analysis of the SERPINB13 gene on chromosome 18q21.3 for 51 samples. Twenty-three tumors were classified as LOH, of which 8 were based on both markers (D18S51 and D18S814) and 15 were based on either one of the two markers. LOH was excluded for 25 cases (17 times based upon two markers), whereas 3 tumor/normal DNA pairs were homozygous for both markers and thus not informative. Statistical analysis using the Mann-Whitney U test revealed a significant association between the presence of LOH at chromosome 18q21.3 and down-regulation of SERPINB13 protein expression in our panel of HNSCC (p=0.006) (Figure 3C). Low SERPINB13 protein expression was also observed in several cases without LOH (Figure 3C, left box), indicating that other mechanisms may also be involved in the regulation of SERPINB13 protein expression in HNSCC. We conclude that the presence of LOH at the SERPINB13 gene on chromosome 18q21.3 is associated with down-regulation of SERPINB13 protein expression in HNSCC.

Down-regulation of SERPINB13 expression is associated with a poor differentiation grade and the presence a lymph node metastasis.

Associations between SERPINB13 expression in the primary HNSCC and various histopathological and clinical parameters of these tumors and patients were analysed (Table 2). A decreased SERPINB13 expression correlated statistically significant with a poor differentiation grade of the primary HNSCC (n=15) as compared to a moderate differentiation grade (n=83) (p=0.001) (Figure 4A). Down-regulation of SERPINB13 expression was also statistically significant associated with both a positive clinical (n=47; p=0.004) and pathological (n=56; p=0.012) N-score, indicating the presence of a LN metastasis based upon palpation and histology, respectively (Figure 4B). No significant associations were observed between SERPINB13 expression and the other histopathological parameters analysed (Table 2). Although no difference was observed for SERPINB13 expression between tumors located in the oral cavity (n=86) versus the oropharynx (n=13) (p=0.563; Tabel 2), SERPINB13 expression was significantly down-regulated in tumors with a sub-localization in the floor of the mouth (n=34) as compared to the tongue (n=34) (p=0.014; Tabel 2).

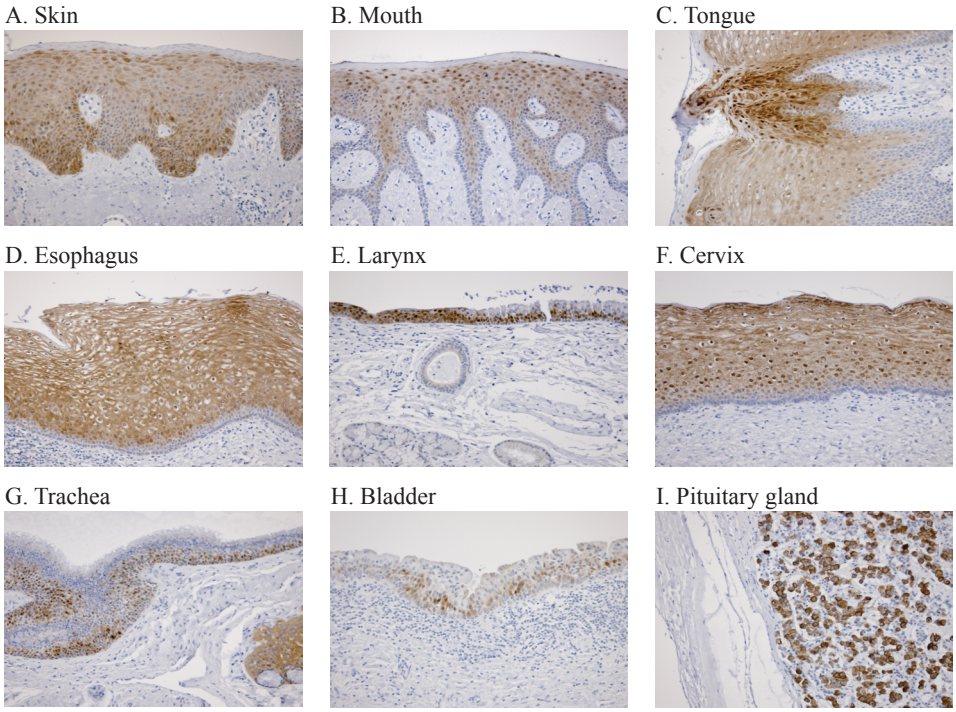
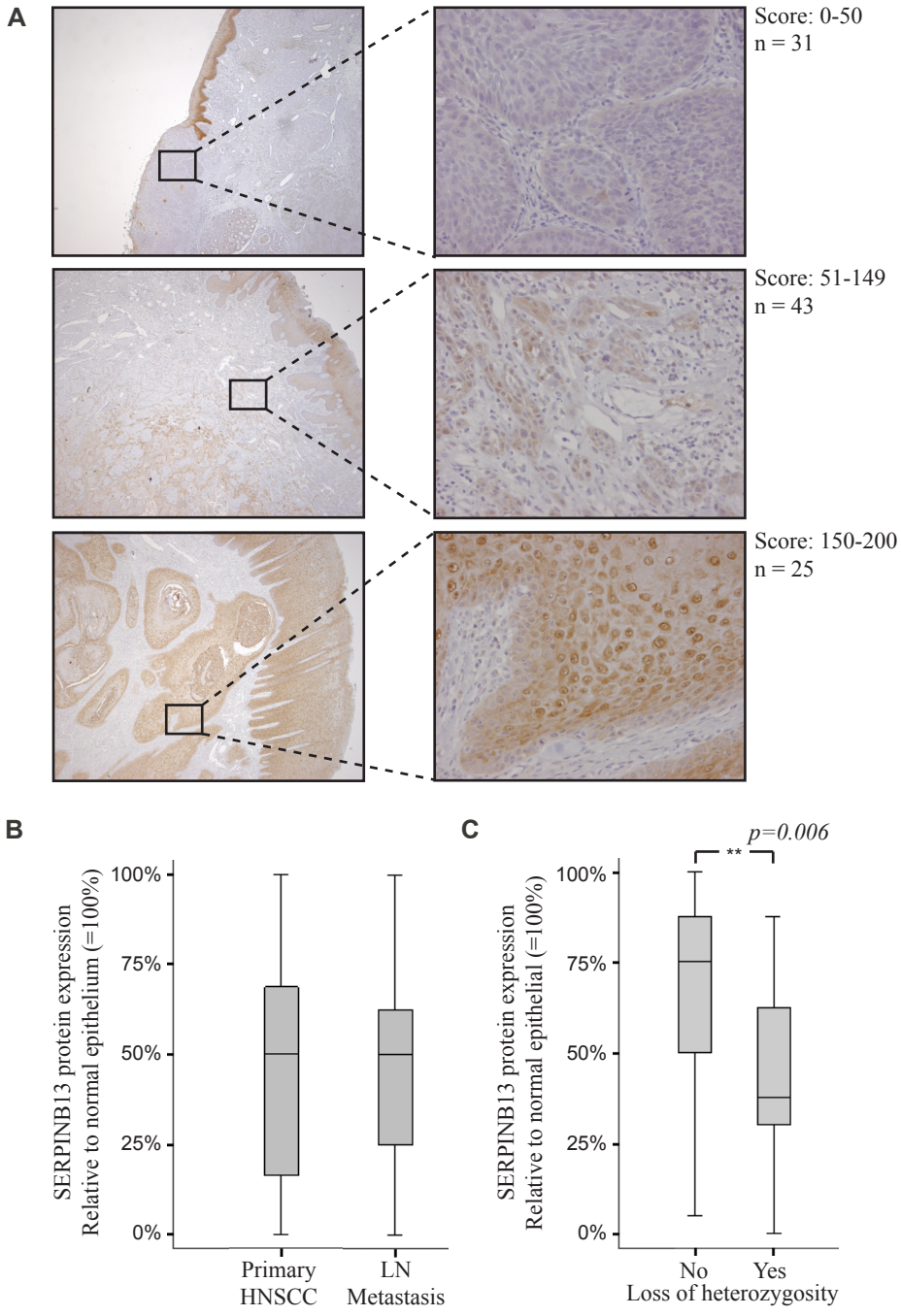


Figure 2. Normal tissue distribution of SERPINB13 protein expression. *Normal tissue distribution of SERPINB13 expression by IHC with mAb 4A9D3. All tissues that expressed SERPINB13 are shown. A-F) Squamous epithelium of the skin, mouth, tongue, esophagus, larynx, and cervix. G) Respiratory epithelium of the trachea. H) Transitional epithelium of the bladder. I) A subset of cells in the pituitary gland.*

Down-regulation of SERPINB13 protein expression in HNSCC is associated with poor clinical outcome.

To investigate whether down-regulation of SERPINB13 protein expression in HNSCC was related to clinical outcome, patients were divided into three groups based upon the SERPINB13 expression score of their HNSCC as previously mentioned. The results showed that down-regulation of SERPINB13 protein expression in HNSCC was statistically significant related with both a decreased overall survival (p=0.018) (Figure 4C) and a decreased disease-free survival (p=0.033) (Figure 4D) of the patients. The area under the ROC curve for prediction of the five years patient survival status by SERPINB13 protein expression score of the tumor was 0.63, with a sensitivity of 64% and a specificity of 60%

Figure 3. SERPINB13 protein expression is down-regulated in HNSCC. *A) Three examples of HNSCC with (almost) total loss, partial loss, and no to marginal loss of SERPINB13 expression in upper, middle, and lower panel, respectively. HNSCC were grouped into three groups (0-50, 51-149, and 150-200) based upon the score of their SERPINB13 expression as compared to SERPINB13 expression in normal epithelium (= 200). Magnification: left panels 20x, right panels 200x. B) Box plot of SERPINB13 protein expression in primary HNSCC (n=99) and their corresponding LN metastases (n=56), relative to SERPINB13 expression in normal epithelium. C) Box plot of SERPINB13 protein expression in HNSCC and corresponding LOH status of SERPINB13 gene (no LOH, n=25 vs. presence of LOH, n=23; p=0.006).* ►


A
Score: 0-50
n = 31
Score: 51-149
n = 43
Score: 150-200
n = 25
B
SERPINB13 protein expression
Relative to normal epithelium (=100%)
100%
75%
50%
25%
0%
Primary
HNSCC
LN
Metastasis
C
p=0.006
**
SERPINB13 protein expression
Relative to normal epithelial (=100%)
100%
75%
50%
25%
0%
No
Yes
Loss of heterozygosity

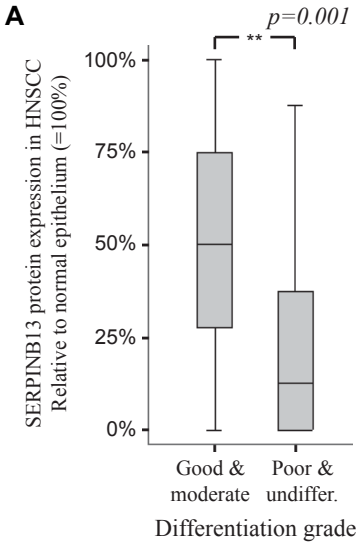
(results not shown). Furthermore, in HNSCC patients, the presence of LOH of the serpin gene cluster at chromosome 18q21.3, including SERPINB13, was found to be associated significantly with a poor overall survival (p=0.042) (Figure 4E).

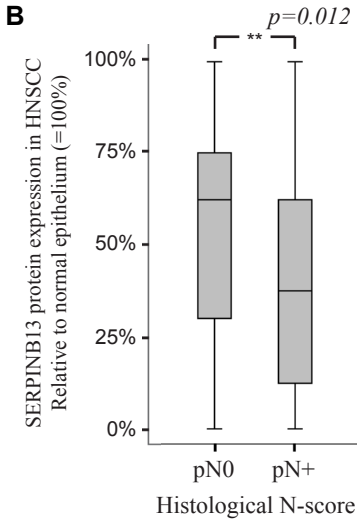
Down-regulation of SERPINB13 protein expression in HNSCC correlated significant with both a poor differentiation grade (p=0.001) and the presence of lymph node metastasis (p=0.012) (Table 2 + Figure 4A-B), whereas tumor grade and lymph node metastasis themselves are known prognostic factors for HNSCC [27,28]. Therefore, multivariable analyses were performed to investigate the influence of these individual parameters on the association of SERPINB13 expression with overall patient survival in our panel of HNSCC. Cox regression analysis revealed a hazard ratio (HR) of 0.62 (95% confidence interval (CI) 0.41-0.93; p=0.021) for the significant association of down-regulation of SERPINB13 expression alone with a decreased overall survival, indicating that a sustained expression of SERPINB13 in HNSCC has a protective effect for these patients regarding their survival time. This relation still remained significantly, independent of differentiation grade (HR 0.63; 95% confidence interval (CI) 0.41-0.97; p=0.037) or treatment of patients with radiotherapy (HR 0.61; 95% CI 0.41-0.92; p=0.019). In contrast, the association between SERPINB13 expression and overall survival was diminished when including lymph node metastasis as an additional factor (HR 0.68; 95% CI 0.45-1.04; p=0.072), whereas the presence of a lymph node metastasis itself remained significantly associated with a poor overall survival (HR 2.17; 95% CI 1.11-4.22; p=0.023). This latter HR indicates an increased risk for shorter survival-time of patients with HNSCC that already metastasized to the lymph nodes as based on histopathological assessment. In conclusion, down-regulation of SERPINB13 protein expression positively associates with a poor clinical outcome.

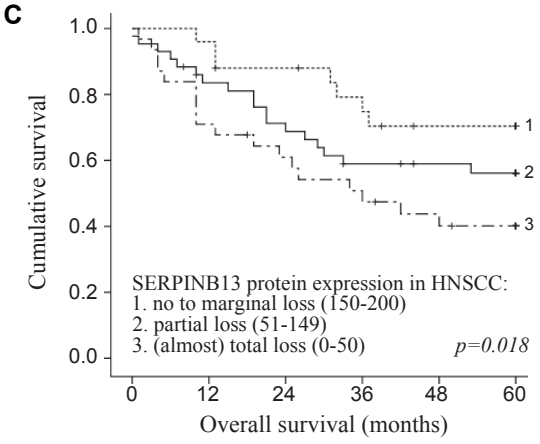
Discussion

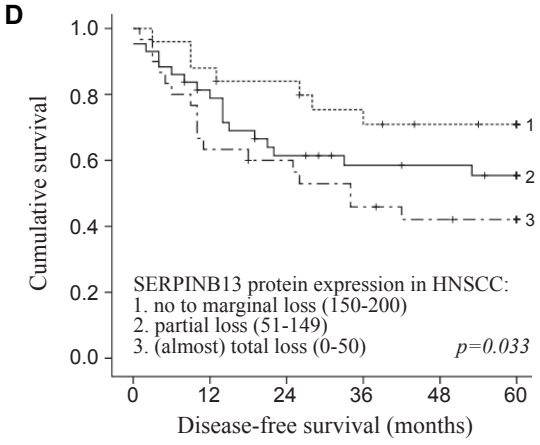
In this study, we report the clinical implications of down-regulation of the intracellular serine protease inhibitor SERPINB13 in HNSCC. We made specific mAbs against SERPINB13 and studied its expression in a cohort of 99 patients with HNSCC. Specifically, we show that down-regulation of SERPINB13 expression is significantly associated with multiple adverse clinical and pathological features, e.g. a poor differentiation grade of the tumors (p=0.001; Figure 4A) and the presence of a lymph node metastasis (p=0.012; Figure 4B). Moreover, SERPINB13 down-regulation is significantly correlated with an increased risk of death (p=0.018; Figure 4C) as well as recurrent disease (p=0.033; Figure 4D), independent of tumor differentiation grade. It has to be noted that the rather low predictive power, specificity, and sensitivity as determined by an ROC curve for the five years patient survival status

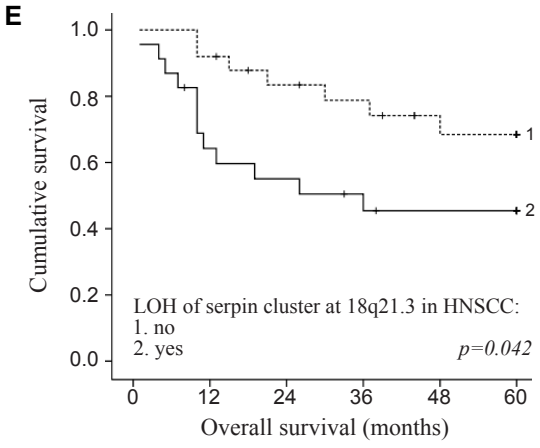
Figure 4. Down-regulation of SERPINB13 expression in HNSCC is associated with poor clinical outcome. *A-B) Box plots of SERPINB13 protein expression in HNSCC and their corresponding A) differentiation grade (good/moderate, n=83 vs. poor/undifferentiated, n=15; p=0.001) and B) histological N-score (pN0, n=43 vs. pN+, n=56; p=0.012). C-D) Kaplan-Meier survival curves of B) overall survival (p=0.018) and C) disease-free survival (p=0.033) of patients with HNSCC and grouped SERPINB13 protein expression: 1. no to marginal loss of SERPINB13 protein expression in HNSCC (score 150-200; n=25), 2. partial loss of SERPINB13 expression (score 51-149; n=43), and 3. (almost) total loss of SERPINB13 expression (score 0-50; n=31). +: censored cases. E) Kaplan-Meier survival curve of patient overall survival and the presence of LOH of the serpin cluster at chromosome 18q21.3 in the HNSCC (no LOH, n=25 vs. presence of LOH, n=23; p=0.042).* ►


A
p=0.001
**
SERPINB13 protein expression in HNSCC
Relative to normal epithelium (=100%)
100%
75%
50%
25%
0%
Good & moderate
Poor & undiffer.
Differentiation grade


B
p=0.012
**
SERPINB13 protein expression in HNSCC
Relative to normal epithelium (=100%)
100%
75%
50%
25%
0%
pN0
pN+
Histological N-score


C
Cumulative survival
1.0
0.8
0.6
0.4
0.2
0.0
SERPINB13 protein expression in HNSCC:
1. no to marginal loss (150-200)
2. partial loss (51-149)
3. (almost) total loss (0-50)
p=0.018
0
12
24
36
48
60
Overall survival (months)


D
Cumulative survival
1.0
0.8
0.6
0.4
0.2
0.0
SERPINB13 protein expression in HNSCC:
1. no to marginal loss (150-200)
2. partial loss (51-149)
3. (almost) total loss (0-50)
p=0.033
0
12
24
36
48
60
Disease-free survival (months)


E
Cumulative survival
1.0
0.8
0.6
0.4
0.2
0.0
LOH of serpin cluster at 18q21.3 in HNSCC:
1. no
2. yes
p=0.042
0
12
24
36
48
60
Overall survival (months)

precludes the use of SERPINB13 as a single marker for individual patients. All together, we conclude that loss of SERPINB13 expression plays a role in the tumor progression and may serve as an additional prognostic marker in HNSCC.

Our observation concerning SERPINB13 expression is consistent with previous studies that demonstrated down-regulation of SERPINB13 mRNA or protein expression in HNSCC [14,16,17]. In the first two studies very small panels of three to five tumors were investigated, while Shellenberger et al. studied SERPINB13 expression by IHC in 60 HNSCC [17]. The latter study, however, focused on the role of SERPINB13 in the suppression of angiogenesis and did not provide any clinical or pathological data of the patients and samples tested. Furthermore, the vast majority of tumors contained only a few scattered cells that showed weak SERPINB13 staining. This is in contrast to our current study where we observed a marked difference in SERPINB13 expression between tumors. To study this difference we assessed the SERPINB13 expression in more detail by combining expression-intensity (as compared to normal epithelium) with the percentage of positive tumor cells. This resulted in a more informative SERPINB13 expression scoring, showing tumors with (almost) total loss (n=31) while other tumors demonstrated no to marginal loss (n=25) (Figure 3A). This difference between the two studies may be explained by the difference in sensitivity between the antibodies used. Our group has extensive experience with the generation of serpin-specific antibodies and their use in immunohistochemical techniques [20]. In the current study we have generated a panel of novel SERPINB13-specific antibodies and specifically selected an antibody (4A9D3) with strong reactivity in immunohistochemistry that, in addition, did not cross-react with any of the other homologous intracellular serpins (Figure 1A). Therefore, our antibody may display a greater sensitivity towards SERPINB13 in immunohistochemical stainings compared to the, commercially available, antibody used in the study of Shellenberger et al.

The SERPINB13 gene is located at chromosome 18q21.3 [14], a region in which LOH is often observed in HNSCC [4,5]. LOH on 18q21.3 was also detected in a significant number of our samples and loss was significantly correlated with SERPINB13 protein down-regulation (p=0.006; Figure 3C), supporting our immunohistochemical results. This suggests that, in the case of LOH, the SERPINB13 gene that is still present on the other allele is not transcribed into a functional protein, probably due to a mutation [29]. Interestingly, 10 out of 13 intracellular serpin family members reside in a cluster on this chromosomal location. The gene area spanned by the two microsatellite markers used in this study contains six other serpin genes, encoding for SERPINB2, -B3, -B4, –B5, -B11 and B12. This suggests that, like SERPINB13, protein expression of these other serpins might also be down-regulated in HNSCC. Indeed, aberrant expression of SERPINB2 and –B5 is associated with progression of HNSCC and patient survival [11,13]. The fact that we identified a significant correlation between LOH on 18q21.3 and overall survival (p=0.042; Figure 4E) suggests that other serpin genes residing within this chromosomal cluster may play a role in HNSCC cancer progression as well.

The balance between protease activity and the presence of its cognate inhibitor is crucial in many biological processes, including tumor progression. In the microenvironment of a tumor, this balance is usually disturbed in favor of an increased proteolytic activity. This can result in degradation of the surrounding extracellular matrix of a tumor and/or induce altered signaling functions, finally leading to tumor progression [30-33]. In the present study, we demonstrate that there is indeed a significant association between down-regulation of the SERPINB13 protein expression and clinical-pathological features that are associated with

Table 2. Associations of SERPINB13 expression in HNSCC with clinical and histopathological parameters (Mann-Whitney U test).

Parameter	*p*-value	
Clinical characteristics		
Sex (male vs. female)	0.652	
Age (≤ 60 vs. > 60 yrs)	0.334	
Tumor localization (oral cavity vs. oropharynx)	0.563	
Tumor sub-location (floor of mouth vs. tongue)	0.014	**
Clinical N-score (N0 vs. N+)	0.004	**
Histopathological characteristics		
Infiltration depth (≤ 10.0 vs. > 10.0 mm)	0.137	
Differentiation grade (good/moderate vs. poor/undifferentiated)	0.001	**
Dysplasia (non/low grade vs. high grade)	0.124	
Vaso-invasion (absent vs. present)	0.387	
Bone-invasion/-erosion (absent vs. present)	0.318	
Perineural growth (absent vs. present)	0.579	
Spidery growth (absent vs. present)	0.433	
Extra nodal growth (absent vs. present)	0.606	
Development of a local recurrence (within 2 yrs)	0.144	
Development of a distant metastasis (within 5 yrs)	0.495	
T-stage (pT1 vs. pT2-4)	0.505	
Histological N-score (N0 vs. N+)	0.012	**

*** : p <0.05 (statistically significant)*

aggressive tumor behavior such as the presence of a LN metastasis and a poor disease-free as well as overall survival of the patients (Figure 4A-D). However, the exact physiological role of SERPINB13 in normal squamous epithelia as well as in squamous cell carcinomas has yet to be elucidated.

In vitro inhibitory profile studies for SERPINB13 using purified proteins revealed the lysosomal cysteine proteases cathepsin K and L as potential target proteases [34,35]. Welss *et al.* showed that over-expression of SERPINB13 protects keratinocytes from UV-induced apoptosis, supposedly by inhibition of unwanted cathepsin L proteolytic activity in the cytoplasm [35]. Increased expression of cathepsin L has been linked to tumor progression and poor clinical outcome for numerous types of cancers, including HNSCC [36-38]. Cathepsin K has only recently been implicated in the turnover of extracellular matrix proteins during cancer invasion [39,40]. Impaired inhibition of cathepsin K and L activity as a result of SERPINB13 down-regulation might result in tumor progression. However, it should be noted that SERPINB13 is a member of the intracellular serpin family and thus resides in the cytoplasm and nucleus. It is not excluded that SERPINB13 might also inhibit a yet unknown target protease or even may perform protease-independent functions in normal epithelial and/or cancer cells. Zhang et al. demonstrated that SERPINB5 (Maspin) blocks neo-vascularization *in vivo*, although this was independent of its inhibitory function [41]. Recently, an inhibitory effect on *in vivo* angiogenesis has also been reported for SERPINB13 [17], though it still has to be elucidated if

this is mediated via a protease or a protease-independent pathway. Whether down-regulation of SERPINB13 expression in HNSCC tips the balance towards more protease activity or simply reflects the state of tumor cell differentiation remains to be elucidated.

The observed expression pattern in normal tissues with restricted expression in respiratory and transitional epithelium (Figure 2G-H) may indicate that SERPINB13 may play a role in tumor progression in the lung and bladder as well. Whether loss of SERPINB13 expression is involved in tumor progression and may identify patients with aggressive tumor behavior at these locations requires further study. In conclusion, down-regulation of SERPINB13 expression identifies HNSCC patients with aggressive tumor behavior and with poor clinical outcome. Therefore, SERPINB13 may act as an important protease inhibitor involved in the progression of HNSCC. This can be of interest for novel therapeutic strategies in patients with HNSCC.

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Chapter 8

Serine protease inhibitor 8 is a novel immunohistochemical marker for neuroendocrine tumors of the pancreas

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Abstract

Objectives / Methods: The intracellular serine protease inhibitor SERPINB8 is expressed by squamous epithelium, monocytes, and a subset of neuroendocrine cells. Using immunohistochemistry, we now have further investigated the expression of SERPINB8 in normal neuroendocrine cells and its potential use as a marker to identify neuroendocrine tumors of the pancreas. *Results:* In normal neuroendocrine tissues, strongest SERPINB8 expression was detected in islets of Langerhans of the pancreas. Moderate SERPINB8 expression was observed in neuroendocrine cells of the thyroid, adrenal cortex, colon, and pituitary gland. Fluorescent double staining revealed that in the pancreas SERPINB8 is specifically expressed by insulin-producing beta cells. In a panel of 20 patients with pancreatic islet cell tumors, however, SERPINB8 was broadly expressed and not restricted to insulinomas. In islet cell tumors, SERPINB8 had a similar diagnostic sensitivity as compared to the widely used neuroendocrine markers chromogranin A and synaptophysin. When SERPINB8 was combined with these two markers even a higher diagnostic sensitivity was reached. In contrast, exocrine adenocarcinomas of the pancreas showed no SERPINB8 expression. *Conclusions:* SERPINB8 is expressed in normal neuroendocrine cells of several organs as well as in neuroendocrine tumors of the pancreas where it can be used as an additional diagnostic immunohistochemical marker.

Abbreviations

mAb	monoclonal antibody
FITC	fluorescein isothiocyanate
HRP	horseradish peroxidase
MEN1	multiple endocrine neoplasm type 1
NEC	neuroendocrine carcinoma
NET	neuroendocrine tumor
PC	prohormone convertase
SERPIN	serine protease inhibitor

Introduction

Neuroendocrine tumors (NETs) constitute a heterogeneous group of neoplasms originating from neuroendocrine cells in different organs, including gastroenteropancreatic neuroendocrine tumors, paragangliomas, and medullary carcinomas of the thyroid [1]. NETs can be divided into functional and non-functional tumors, of which only the former secretes its specific original hormone peptide(s), *e.g.* insulin production by insulinomas. This disparity is used for diagnosis and characterization of NETs because most well-differentiated NETs still produce their parent hormone(s), while poorly differentiated NETs often loose this expression. Furthermore, also sensitive general neuroendocrine cell markers are used for diagnosis of NETs, *e.g.* chromogranin A and synaptophysin, but some NETs are even negative for these markers [2].

The production of hormones is the major function of neuroendocrine cells, via which diverse processes are regulated throughout the human body. Upon production, hormones are stored in granules of neuroendocrine cells to be secreted after a stimulus. These hormones are initially produced as inactive pro-peptides and need to be cleaved to obtain active functional hormones. The family of subtilisin-like proprotein convertases strictly regulates this process, which takes place mainly in granules of the secretory pathway [3]. Up to now, nine mammalian proprotein convertases are known, including furin, prohormone convertase (PC) 1/3, and PC2. Furin is a ubiquitously expressed proprotein convertase and is involved in many biological processes as it activates large numbers of proprotein substrates, including growth factors and blood clotting factors [4]. PC1/3 and PC2 are predominantly expressed in neuroendocrine cells and are known as the major prohormone convertases [5].

The activity of intracellular proteases, such as proprotein convertases, is strictly regulated to control protease activation and to prevent unwanted cellular damage [6]. To date, only few endogenous inhibitors of proprotein convertases have been identified. Furin is strongly inhibited by the intracellular serine protease inhibitor SERPINB8 (protease inhibitor 8 (PI-8) / cytoplasmic anti-protease 2 (CAP2)) *in vitro* [7,8]. Although intracellular serpins are widely expressed among human tissues, each of them has its own restricted cellular distribution[9]. This restricted cellular expression partly reflects their function in controlling activity of a target protease in these specific cells. In a previous study, we have demonstrated that SERPINB8 is strongly expressed by neuroendocrine cells of the pituitary gland, the digestive tract, and the pancreas [10]. This indicates a possible protective role for SERPINB8 against unwanted proteolysis in these neuroendocrine cells.

In the present study, we have investigated the expression of SERPINB8 in normal neuroendocrine cells in more detail. The strong and specific expression by insulin-producing beta cells in the normal pancreas prompted us to study the value of SERPINB8 as a diagnostic immunohistochemical marker in a panel of pancreatic islet cell tumors.

Materials and methods

Patients and tissue specimens

Formalin-fixed paraffin-embedded biopsies from normal tissues, pancreatic islet cell tumors, and adenocarcinomas of the pancreas were obtained from the tissue bank of the Department of Pathology / UMCU Biobank, UMC Utrecht, and used in line with the code

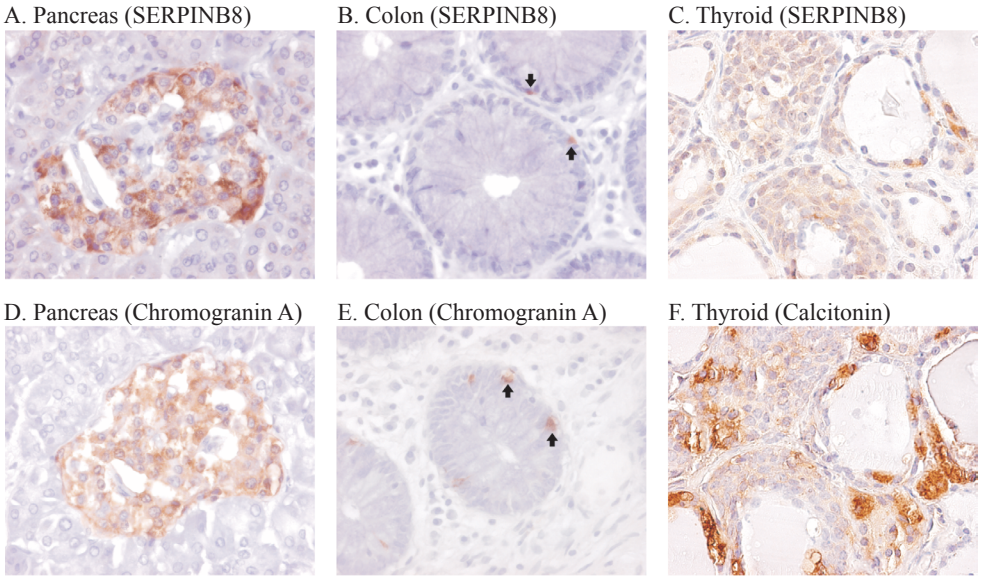

Figure 1. SERPINB8 is expressed in neuroendocrine cells in various organs. *Expression of SERPINB8 (A-C), chromogranin A (D, E), or calcitonin (F) in neuroendocrine cells in serial sections of pancreas (A, D), colon (B, E), and thyroid (C, F) (hematoxlin counterstained, original magnification x 300).*

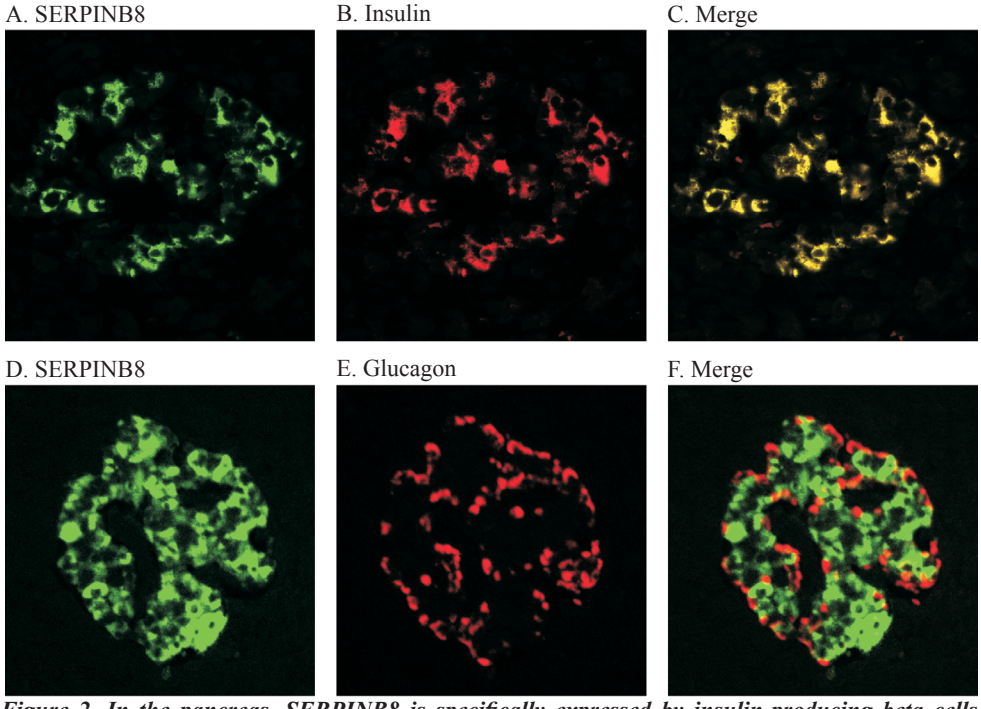

Figure 2. In the pancreas, SERPINB8 is specifically expressed by insulin-producing beta cells. *Fluorescent double staining of islets of Langerhans in the pancreas showing co-expression of SERPINB8 (A and D: FITC) with insulin (B: Texas Red), but not with glucagon (E: Texas Red). C and F show merged images (original magnification x 320).*

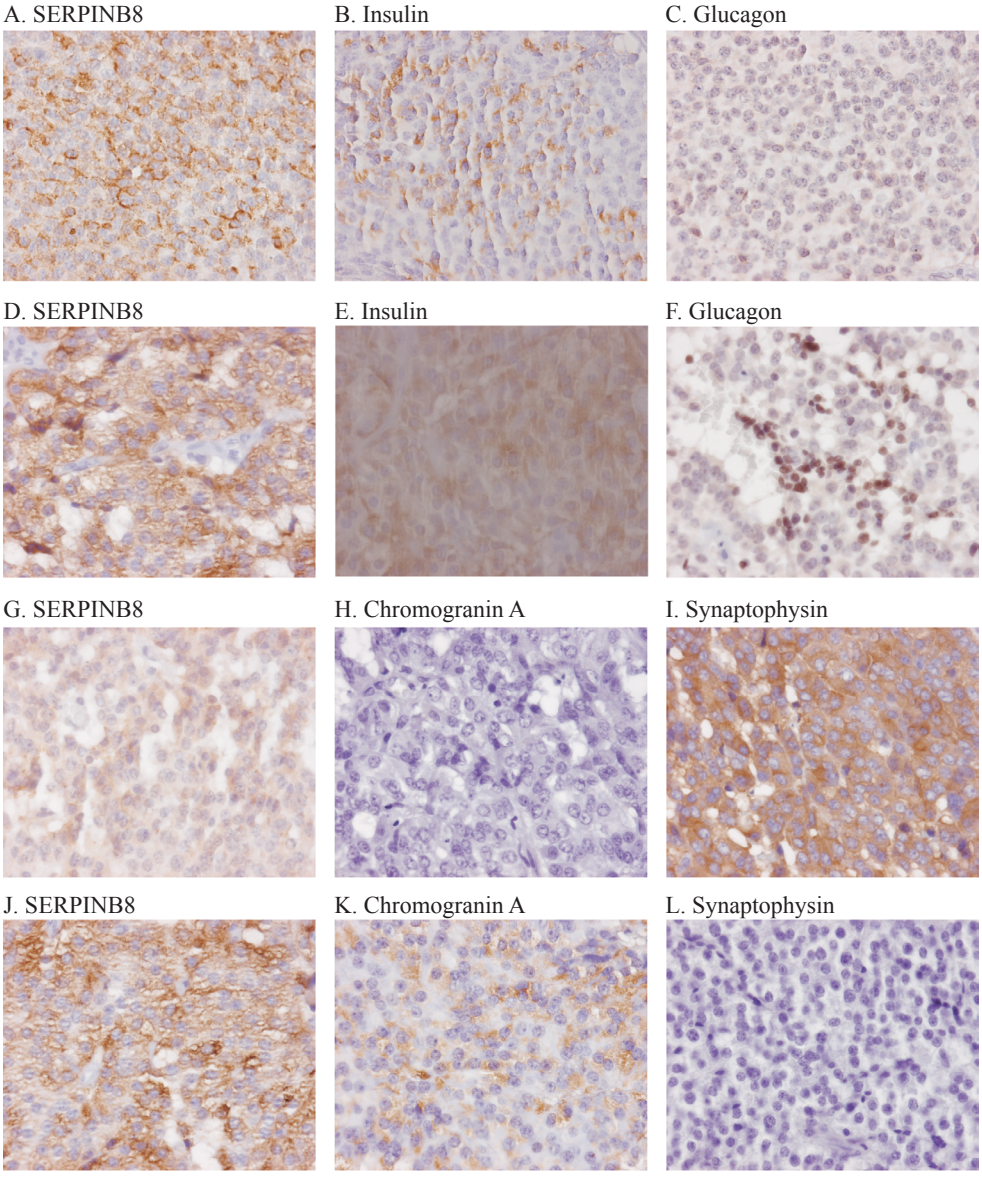

Figure 3. SERPINB8 expression in pancreatic islet cell tumors is complementary to the neuroendocrine markers chromogranin A and synaptophysin. *Staining of islet cell tumors of the pancreas for SERPINB8 (A, D, G, J) and the endocrine markers insulin (B, E), glucagon (C, F), chromogranin A (H, K), and synaptophysin (I, L). Examples for four different tumors are shown (A-C, D-F, G-I, J-L, corresponding with patient nr. 13, 7 (NEC), 6 (NET), and 7 (NEC), respectively) with a strong SERPINB8 expression in all cases and variable staining for the other markers (hematoxylin counterstained, original magnification x 300).*

"Proper Secondary Use of Human Tissue" as installed by the Federation of Biomedical Scientific Societies (www.federa.org/?s=1&m=78&p=&v=4). Material and patient characteristics are further shown in Tables 1. Endocrine pancreas tumors were classified according the recent WHO criteria [11] including size, cell differentiation, angio-invasion, proliferation index, metastatic spread, hormone expression, and functional activity, and finally diagnosed as well-differentiated neuroendocrine tumors (NETs), well-differentiated neuroendocrine carcinomas (NECs), or poorly differentiated NECs.

Antibodies and reagents

The mAb anti-PI-8-k was raised against *Pichia pastoris*-expressed human SERPINB8 as described previously [12]. The following antibodies were obtained from Dako (Glostrup, Denmark): polyclonal rabbit anti-human insulin, rabbit anti-human glucagon, rabbit anti-human chromogranin A, rabbit anti-human calcitonin, monoclonal mouse anti-human synaptophysin (clone SY38), HRP-conjugated rabbit anti-mouse Ig, and HRP-conjugated swine anti-rabbit Ig. Biotin-conjugated goat anti-rabbit IgG and horse anti-mouse IgG were purchased from Vector Laboratories (Burlingame, CA), and HRP-labeled streptavidin was obtained from Immunotech (Marseille, France). Tyramide amplification buffer, biotin-labeled tyramide, FITC-labeled tyramide, and streptavidin-conjugated Texas Red were purchased from Perkin Elmer (Boston, MA).

Immunohistochemistry

Four μm thick sections were cut and mounted on silane-coated glass slides. After deparaffinization and blocking endogenous peroxidase activity, the tissue sections were subjected to antigen retrieval by boiling in 10 mM sodium-citrate buffer, pH 6.0, for 10 min in a microwave. Subsequently, the tissue slides were washed with PBS, 0.05% (v/v) Tween-20. Primary antibodies were diluted in PBS, 1% (w/v) BSA as follows: PI-8-k 0.93 μg/ml, synaptophysin 1:1000, insulin 1:2000, glucagon 1:1000, chromogranin A 1:500, and calcitonin 1:4000, and incubated for 1 hour. Subsequently, the tissue slides were incubated with a biotin-conjugated antibody: horse anti-mouse IgG (1:250) or goat anti-rabbit IgG (1:250), followed by HRP-labeled streptavidin (1:400). After washing with PBS, SERPINB8 or hormone markers were visualized using 3,3'-diaminobenzidine (0.6 mg/ml). Slides were counterstained with haematoxylin. Appropriate positive and negative controls were used throughout.

Percentages of positive tumor cells were evaluated semi quantitatively independently by 2 observers. In case of disagreement, the observers reanalyzed the staining results until they reached consensus. According to the number of tumor cells staining positively, positive cases in the panel of endocrine pancreas tumors were divided into three categories: <5%, 5-50%, and >50% of the tumor cell population staining positive for the marker tested. Also the intensity of the staining was scored and compared to the staining-intensity of non-neoplastic neuroendocrine cells present in the same slide: stronger (++), similar (+), weaker (+/-), or negative (-).

Immunofluorescent double staining

Immunofluorescent double staining was performed on normal pancreas tissue slides for SERPINB8 and either insulin or glucagon. Tissue slides were initially pre-treated according to the above protocol. Next, the tissue slides were incubated for 1 hr with rabbit anti-human

insulin (1:2000) or rabbit anti–human glucagon (1:1000), followed by incubation with HRP-conjugated swine anti-rabbit Ig (1:50 in PBS with 10% normal human serum). After washing with PBS, 0.05% (v/v) Tween-20, slides were pre-incubated for 3 min with a tyramide amplification buffer, directly followed by biotin-conjugated tyramide (1:50) for 10 min. Subsequently, Texas Red-labeled streptavidin was used for 30 min to visualize the presence of insulin or glucagon. Then, the tissue slides were blocked using 10% normal rabbit serum (Dako) for 30 min, and peroxidase activity was blocked again. Next, SERPINB8 was stained with mouse anti-human PI-8-k for 1 hr, followed by peroxidase-labeled rabbit-anti-mouse Ig (1:50 in PBS with 10% normal human serum). Slides were washed and pre-incubated with amplification buffer, followed by tyramide-FITC (1:50). Finally, slides were washed again and mounted in Vectashield (Vector).

Results

SERPINB8 is strongly expressed by beta cells of the pancreas.

SERPINB8 is known to show a restricted cellular expression in several human organs. Besides monocytes and squamous epithelial cells (*e.g.* in the oral cavity, esophagus, and epidermis), SERPINB8 is specifically expressed by neuroendocrine cells in the endocrine pancreas (islets of Langerhans), pituitary gland, and digestive tract [10]. In the present study, we have investigated the expression of SERPINB8 in neuroendocrine cells in more detail. Staining of SERPINB8 and the neuroendocrine cell marker chromogranin A or calcitonin on serial sections of various normal tissues revealed strong expression of SERPINB8 in neuroendocrine cells in Langerhans islets of the pancreas (Figure 1A+D) and moderate expression by neuroendocrine cells in the colon (Figure 1B+E), pituitary gland, cortex of the adrenal gland, and C-cells in the thyroid (Figure 1C+F). Neuroendocrine cells in the small intestine, stomach, parathyroid, and adrenal medulla did not express SERPINB8. Islets of Langerhans consist of several neuroendocrine cells types of which the glucagon-producing alpha cells and insulin-producing beta cells form the majority. Fluorescence double staining of SERPINB8 with either insulin or glucagon demonstrated that SERPINB8 specifically co-localized with insulin (Figure 2A-C), but not with glucagon (Figure 2D-F). These data indicate that SERPINB8 expression by neuroendocrine cells is restricted to certain organs of which the strongest SERPINB8 expression was detected in insulin-producing beta cells in the islets of Langerhans in the pancreas.

SERPINB8 expression in pancreatic islet cell tumors

Next, we investigated whether the restricted and strong SERPINB8 expression in the pancreas was retained in islet cell tumors. A panel of pancreatic tumors, containing both endocrine and exocrine neoplasms, was stained for SERPINB8. Seventy-five percent (18 out of 24) islets cell tumors, including metastases, showed clear SERPINB8 expression (Table 1). In all these tumors, the intensity of SERPINB8 expression was similar or even upregulated as compared to the endogenous expression level of SERPINB8 in non-neoplastic islet cells, which were present in the surrounding normal pancreatic tissue. The panel of islet cell tumors consisted of both neuroendocrine carcinomas (NECs; n=15) and neuroendocrine tumors (NETs; n=5). All NETs expressed SERPINB8, whereas 67 percent of the NECs showed clear SERPINB8 expression. From patient 6 both a NET and a NEC were obtained, of which the

Table 1. Almost all pancreatic islet cell tumors express SERPINB8. *Patients' characteristics and expression of the neuroendocrine proteins SERPINB8, insulin, glucagon, chromogranin A, and synaptophysin in 20 pancreatic islet cell tumors and their corresponding metastases. Stainings were scored on intensity (++, +, +/-, -) and percentage of tumor cells with expression (<5%, 5-50%, >50%). IHC indicates immunohistochemistry; NEC, neuroendocrine carcinoma; NET, neuroendocrine tumor; MEN1, multiple endocrine neoplasm type 1; ND, not determined.*

Pat. nr.	Sex	Age (y)	Tissue origin	Diagnosis	Tumor size (cm)	Functional (clinically)	MEN1
1	F	42	Pancreas	NEC, well differentiated	2.1	N.D.	No
1	F	46	Liver	Metastasis	6.5	No	No
2	F	42	Pancreas	NET-benign (insulinoma)	0.9	Yes (insulinoma)	No
3	F	56	Pancreas	NEC, well differentiated	8.0	No	No
4	M	31	Pancreas	NEC, well differentiated	4.5	No	Yes
5	M	54	Pancreas	NEC, well differentiated	> 10.0	No	No
5	M	54	Lymph node	Metastasis	N.D.	No	No
6	F	39	Pancreas	NET-uncertain behavior	8.0	No	Yes
6	F	39	Pancreas	NEC, well differentiated	Multiple nodes	Yes (glucagonoma)	Yes
7	M	64	Pancreas	NEC, well differentiated	2.0	Yes (insulinoma)	No
7	M	64	Lymph node	Metastasis	2.0	N.D.	No
8	F	73	Pancreas	NET-benign (insulinoma)	1.5	Yes (insulinoma)	No
9	M	61	Pancreas	NEC, well differentiated	2.0	N.D.	No
10	M	54	Pancreas	Hyperplasia with pancreatitis	N.D.	N.D.	No
11	M	33	Pancreas	NEC, well differentiated	4.5	No	No
12	M	62	Pancreas	NET-benign (insulinoma)	1.4	Yes (insulinoma)	No
13	F	5	Pancreas	NEC, well differentiated	3.6	Yes (insulinoma)	No
14	M	38	Pancreas	NET-uncertain behavior	1.2	Yes (insulinoma)	No
15	M	10	Pancreas	NEC, well differentiated	9.5	No	No
16	F	48	Pancreas	NEC, poorly differentiated	4.0	No	No
17	M	67	Pancreas	NEC, well differentiated	14.0	Yes (glucagonoma)	No
18	M	74	Pancreas	NEC, well differentiated	4.8	N.D.	No
19	M	31	Pancreas	NEC, well differentiated	3.0	No	Yes
20	F	48	Pancreas	NEC-uncertain behavior	2.0	N.D.	No

Pat. nr.	Score of IHC stainings				
	SERPINB8	**Insulin**	**Glucagon**	**Chromogranin**	**Synaptophysin**
1	- (0)	- (0)	- (0)	+ (>50)	+ (>50)
1	- (0)	- (0)	- (0)	- (0)	- (0)
2	++ (>50)	+ (5-50)	+ (>50)	++ (>50)	++ (>50)
3	+ (>50)	- (0)	- (0)	++ (>50)	++ (>50)
4	++ (>50)	- (0)	+/- (<5)	+ (>50)	+ (>50)
5	+ (5-50)	- (0)	- (0)	- (0)	- (0)
5	+ (5-50)	- (0)	+ (5-50)	+ (>50)	++ (>50)
6	+ (>50)	- (0)	- (0)	- (0)	+ (>50)
6	- (0)	- (0)	+ (>50)	++ (>50)	++ (>50)
7	++ (>50)	+ (>50)	+ (5-50)	+ (5-50)	- (0)
7	++ (>50)	- (0)	- (0)	+ (>50)	++ (>50)
8	++ (>50)	+ (>50)	+/- (<5)	+ (>50)	+ (>50)
9	+ (>50)	- (0)	- (0)	+ (>50)	+ (5-50)
10	++ (>50)	+ (>50)	+ (5-50)	++ (>50)	+ (>50)
11	++ (>50)	- (0)	+/- (<5)	+ (>50)	++ (>50)
12	++ (>50)	+ (>50)	+ (>50)	+ (5-50)	+ (>50)
13	++ (>50)	+ (5-50)	- (0)	++ (>50)	++ (>50)
14	++ (>50)	+ (>50)	N.D.	+ (5-50)	++ (>50)
15	+ (>50)	- (0)	+ (5-50)	+ (>50)	+ (>50)
16	+ (>50)	- (0)	+ (>50)	++ (>50)	++ (>50)
17	+ (5-50)	- (0)	+ (>50)	++ (>50)	+ (>50)
18	- (0)	- (0)	+/- (<5)	++ (>50)	+ (>50)
19	+/- (<5)	- (0)	+ (5-50)	+ (5-50)	+ (>50)
20	- (0)	- (0)	+ (>50)	+ (>50)	N.D.

NET was SERPINB8 positive, whereas the NEC did not express SERPINB8. The only poorly differentiated NEC in the tested panel (patient 16), however, still expressed SERPINB8. Two lymph node metastases of SERPINB8-positive islet cell tumors (of patients 5 and 7) expressed SERPINB8 as well, suggesting that SERPINB8 expression is not changed in-between these different tumor-stages. No correlation was found between SERPINB8 expression and the familiar multiple endocrine neoplasm type 1 (MEN1) syndrome (patients 4, 6, and 19) (Table 1). Thus, SERPINB8 is strongly expressed in a high percentage of islet cell tumors consisting of both NETs and NECs, and SERPINB8 expression remained unaltered during metastasis towards lymph nodes.

SERPINB8 is a sensitive marker for endocrine pancreas tumors

The strong and specific expression by insulin-producing beta cells in the normal pancreas prompted us to study the relation between SERPINB8 expression and insulin or glucagon hormone production by the tumor cells. Six patients in our panel had a clinically functional insulinoma and indeed all showed local insulin production by the tumor cells (Table 1 + Figure 3B&E). In addition, all these insulinomas showed a very strong SERPINB8 expression in a high percentage (>50%) of tumor cells (Table 1 + Figure 3 A&D). In contrast to the normal pancreas, however, SERPINB8 expression was not restricted to insulin producing cells. Of 11 tumors that expressed substantial (> 5%) amounts of glucagon, as determined by immunohistochemistry, eight expressed the SERPINB8 protein as well (Table 1 + Figure 3 A&C, D&F). In one of the two functional glucagonomas the tumor was negative for SERPINB8 (patient 6), whereas the other one was SERPINB8-positive (patient 17).

Because most islet cell tumors expressed SERPINB8 abundantly, we wondered if SERPINB8 could be used as a general diagnostic marker for pancreatic islet cell tumors. We compared the sensitivity of SERPINB8 as a tumor marker with the expression of the two sensitive and widely used neuroendocrine cell-tumor markers synaptophysin and chromogranin A. Twenty-one and 20 out of 24 pancreatic islet cell tumors (including metastasis) were positive for chromogranin A and synaptophysin, respectively, indicating a strong correlation with SERPINB8 expression (Table 1). Most tumors that lost expression of either synaptophysin, or chromogranin A, or SERPINB8, still expressed the other two markers (Figure 3G-I: patient nr. 6 (NET) and Figure 3J-L: patient 7 (NEC)). Thus, SERPINB8 expression in pancreatic islet cell tumors is complementary to the general neuroendocrine tumor markers chromogranin A and synaptophysin. Moreover, 90 percent (18 out of 20) of the primary islet cell tumors and their corresponding metastases expressed either chromogranin A or synaptophysin whereas 95 percent (19 out of 20) expressed chromogranin A, synaptophysin, or SERPINB8 (Table 1). Only in the case of a liver metastasis all three markers were negative (patient 1). None of 10 exocrine pancreatic adenocarcinomas tested expressed SERPINB8 (data not shown), indicating that in the pancreas SERPINB8 expression is specific for islet cell tumors. These data demonstrate that SERPINB8 can be used as an additional, sensitive, and complementary immunohistochemical identification marker for neuroendocrine tumors of the pancreas.

Discussion

In this study, we have shown expression of the intracellular serine protease inhibitor SERPINB8 in normal neuroendocrine cells in various organs as well as in the majority

of neuroendocrine tumors of the pancreas. In normal tissues, the strongest SERPINB8 expression was detected in beta cells in the pancreatic islets of Langerhans as was confirmed by immunofluorescent double labeling with insulin. This expression in beta cells is in contrast to our previous study were we reported SERPINB8 expression in the glucagon-producing alpha cells [10]. There, we stained sequential slides while in the current study a more reliable immunofluorescent double labeling was performed. In contrast with its restricted expression by insulin-producing beta cells in normal pancreatic tissue, SERPINB8 was broadly expressed by islet cell tumors. Although all insulinomas strongly expressed SERPINB8, this serpin was also detected in other types of islet cell tumors, including a functional glucagonoma. Apparently, SERPINB8 expression in normal pancreatic neuroendocrine cells can differ from that in their corresponding endocrine pancreas tumor cells. The cellular origin of islet cell tumors, however, is still under debate. An islet cell tumor may originate from an already differentiated cell, e.g. a beta cell. Alternatively, such a neoplasm may grow from a less differentiated precursor cell or even a pluripotent cell in the pancreatic ductal / acinar system [13,14]. Our results indicate that islet cell tumors derived from non-beta cells may acquire SERPINB8 expression during tumorigenesis.

Diagnosis of neuroendocrine tumors largely depends on the functionality of these tumors. Functional tumors are mostly diagnosed on the basis of clinical symptoms and elevated blood hormone levels, whereas most non-functional tumors are not diagnosed until histopathological assessment [15]. Chromogranin A and synaptophysin are widely used as standard markers for immunohistochemical identification of neuroendocrine tumors. Our data indicate that SERPINB8 can be used as an additional immunohistochemical diagnostic marker for pancreatic islet cell tumors. Inclusion of SERPINB8 as additional marker besides chromogranin A and synaptophysin resulted in a slightly improved diagnosis of these primary islet cell tumors and corresponding metastases from 90 to 95 percent. Chromogranin A can also be used as a serum marker, although the predicting value of non-functional endocrine pancreas tumors is rather low [16]. Recently, SERPINB8 is found to be released following platelet activation [8]. Although this has not been studied yet for neuroendocrine cells, it may be that SERPINB8 is also released by neuroendocrine cells and thus can serve as a serum marker as well for (non-functional) neuroendocrine tumors.

Several members of the family of intracellular serpins are also used as a marker for disease progression. For example, elevated serum levels of SERPINB3 (SCCA-1) and SERPINB4 (SCCA-2) correlate with lymph node metastasis, tumor stage/volume, recurrence, and shorter disease-free survival of patients with SCC of the uterine cervix and head and neck region [17,18]. SERPINB9 expression is associated with high grade lymphomas [19] and predicts clinical outcome in melanoma patients treated with immunotherapy [20]. In the present study, we have shown that in the majority of endocrine pancreatic tumors and corresponding lymph node metastasis SERPINB8 expression remains generally unaltered as compared with normal islet cells. In certain cases SERPINB8 expression is even upregulated, including four out of five NETs. Whether or not SERPINB8 expression in pancreatic islet cell tumors correlates with prognosis should be investigated in a larger cohort including clinical follow-up.

We hypothesize that a strong cytoplasmic expression of SERPINB8 may protect these tumor cells against unwanted proteolysis by a, yet unknown, protease. A similar function is described for other intracellular serpins, *e.g.* SERPINB9 protects cytotoxic lymphocytes from their endogenous GrB [21] and is highly expressed in several lymphoma types [19]. SERPINB6 protect mast cells and their tumorous counterparts from leakage of beta-

tryptase monomers from secretory granules [22]. To date, several target proteases have been described for SERPINB8, *i.e.* furin [7], thrombin [23], subtilisin A [12], chymotrypsin [24], factor Xa [12], and trypsin [12]. Except for furin [8], however, the interaction of SERPINB8 with these target proteases has only been demonstrated by using purified proteins. Whereas the prototypical mammalian kexin/subtilisin-like endoproteinase furin is ubiquitinuously expressed, the expression of SERPINB8 is restricted to squamous epithelium, monocytes, and several neuroendocrine cells [10]. This suggests that, besides furin, SERPINB8 may inhibit even another, yet unidentified, target protease with a similar cellular distribution as SERPINB8. The expression of SERPINB8 in the insulin-producing beta cells and calcitonin-producing C-cells suggests that SERPINB8 can inhibit a target protease in these cells and may reflect a function of SERPINB8 in the processing of insulin and calcitonin, respectively. Whether SERPINB8 plays a role in hormone processing, protects against unwanted proteolysis, or is involved in other processes in neuroendocrine cells and pancreatic islet cell tumors requires further study.

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Chapter 9

General discussion

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General discussion

The interaction of cells of the immune system with transformed cells is a complex mechanism that is of high importance to prevent tumor formation and to fight against cancer. Granzymes are involved in a major pathway of cytotoxic lymphocytes to kill tumor and virus-infected cells. In this thesis, diverse aspects of certain human granzymes have been investigated, such as cellular expression, substrates, and inhibitors. Our findings have resulted in a better understanding of how immune cells fulfill their cytotoxic function, which may eventually lead to novel therapeutic approaches. Secondly, protein expression of serpins has been investigated in specific types of tumors to determine if these protease inhibitors can affect tumor progression. This knowledge may be used for future treatments of patients with these types of cancer. In addition, the studied granzymes and serpins might be potential diagnostic or even prognostic markers for inflammatory diseases and cancer. The current findings as well as some future study perspectives are further discussed in this chapter.

Cellular expression of granzymes

GrA and GrB are widely used as markers for cytotoxic lymphocyte-mediated diseases, e.g. transplant rejections, viral infections, arthritis, and lymphomas [1-4]. However, human GrB has also been reported to be expressed by basophils and plasmacytoid dendritic cells [5,6], whereas its expression in neutrophils remains controversial [7-11]. As GrB is used as a marker for cytotoxic lymphocytes, but its expression is not restricted to these cells, it is of importance to first completely unravel which cell types express this specific granzyme. In chapter three, we demonstrated that activated human mast cells express and release GrB, suggesting a role for this protease as novel mediator in the diverse immunological actions of mast cells. Lesional mast cells of patients with cutaneous or systemic mastocytosis abundantly expressed GrB. In concordance, Pardo et al. recently showed that GrB is expressed by murine mast cells [12]. Thus, the novel insights about the cellular expression pattern of GrB challenge its specificity as a marker for cytotoxic lymphocytes.

Whether human GrM is a suitable marker for cytotoxic lymphocytes and for diseases in which these cells are involved is currently unknown. The cellular protein expression of GrM has been subject of only two studies so far, demonstrating that GrM is highly expressed by NK cells, NKT cells, and γδ T cells, whereas its expression by $CD8^+$ T cells remains controversial [13,14]. Whether human GrM is also expressed by non-lymphocytes, e.g. mast cells, has not been investigated yet. Besides the possible use of GrM as a marker for specific subsets of cytotoxic lymphocytes, elucidation of its cellular expression pattern provides more insight into the cell-populations that can execute a GrM-mediated action. In chapter four, we show by flow cytometry with a novel specific mAb that GrM is not only expressed by NK cells, NKT cells, and γδ T cells, but also by $CD8^+$ T cells and slightly by $CD4^+$ T cells. Of the determined T cell subsets, differentiated effector $CD8^+$ cells showed highest GrM protein expression, which is in concordance with the GrB and perforin expression in these cells. However, we determined that GrM expression is differentially regulated as compared to GrB. Because GrM also induces a unique cell death pathway [15,16], these data suggest that GrM is not just a back-up mechanism for the other granzymes, but rather points to a specialist function in specific processes. Thus, human GrM is not only expressed by cytotoxic lymphocytes of the innate immunity, but also by T cell subsets of the adaptive immune system. Our current findings suggest that both effector pathways of the immune system can mediate the unique

GrM-induced target cell death route.

Extracellular granzymes

The physiological function of GrB released from cell types that do not express perforin, e.g. mast cells (chapter three), remains an intriguing question. Interestingly, GrB released by murine mast cells has been suggested to contribute to perforin-independent cell death by anoikis and an increased vascular permeability [12]. Evidence for non-cytotoxic and/or extracellular functions of human GrA and GrB is emerging lately, e.g. degradation of multiple extracellular matrix proteins [17,18]. In addition, elevated concentrations of GrA, GrB, and GrK were detected in tissue fluids during diverse inflammatory processes, like viral infections and arthritis [17]. In some of these diseases, the three granzymes can even be used as prognostic markers because their protein levels in the circulation associate with disease severity. Whether this can be extrapolated to GrH and GrM is currently unknown. Moreover, it is completely unknown if human GrM and GrH are involved in pathological processes.

To study the presence of GrM in tissue fluids, we generated a panel of novel monoclonal antibodies against human GrM and subsequently developed a sandwich ELISA for this serine protease (unpublished results). Using this ELISA, soluble recombinant human GrM was detected into the low nanogram range in phosphate-buffer and cross-reactivity with recombinant human GrB and GrK was excluded (Figure 1A). In addition, native GrM could be detected in cell lysate of the human NK cell line KHYG-1, and not in control HeLa and Jurkat cell lysates (Figure 1B). However, recombinant GrM antigen levels as recognized by our sandwich ELISA were strongly decreased after prior incubation with serum and plasma (Figure 1C). This may be explained by binding of GrM to a specific extracellular inhibitor or other non-inhibitory plasma components such as proteoglycans, thereby blocking an epitope recognized by one of the antibodies. Such a mechanism has previously been demonstrated for human GrA [19].

Some future perspectives will be discussed that may lead to the generation of a functional sandwich ELISA to detect GrM levels in the circulation. First the factor that disturbs detection of GrM in serum and plasma needs to be identified. Second, the sandwich ELISA for GrM has to be re-optimized, e.g. testing combinations of antibodies against GrM with antibodies against the GrM-binding plasma component. A functional GrM sandwich ELISA can finally be used to detect GrM levels in the circulation of patients with inflammatory diseases and tumors, similar as was done for GrA and GrB. Such an assay can give insight if human GrM plays a role in these diseases. If so, GrM levels may even be used as a diagnostic or prognostic marker.

When GrM levels in the circulation or other body fluids are indeed increased in inflammatory settings, it is intriguing to investigate if GrM still harbors proteolytic activity in the extracellular compartment. Currently, it is unknown if GrM can hydrolyze extracellular substrates, like GrA and GrB [17], and thereby contribute to such inflammatory process. Two extracellular serpins are already known to bind to and inhibit GrM, i.e. SERPINA1 and SERPINA3 [20]. In addition, the novel identified intracellular GrM-inhibitor SERPINB4 (chapter six) is also present in the circulation at increased levels in patients with a squamous cell carcinoma [21,22], thereby possibly inhibiting the activity of extracellular GrM. A similar mechanism has already been described for GrB and its inhibitor SERPINB9 in patients with CMV infection [23]. If GrM is present in one or more body fluids and a fraction is still proteolytic active, although the presence of extracellular serpins, the generation of a specific

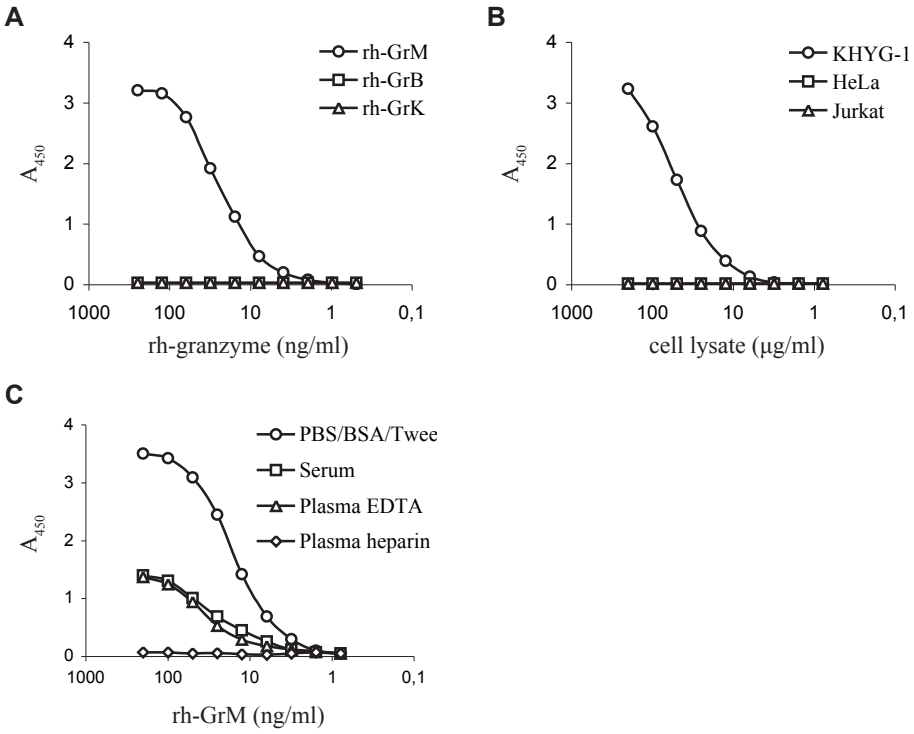

Figure 1. Detection of soluble native and recombinant human GrM, using a specific and sensitive sandwich ELISA. *A) A sandwich ELISA for GrM was developed with mAbs 4E2D5 and biotin-labeled 4B2G4, detecting recombinant human (rh) GrM into the low nanogram-range. Cross-reactivity with soluble rh-GrB and rh-GrK was excluded. B) Native GrM present in the human NK cell line KHYG-1 was detected in cell lysate, and not in control HeLa and Jurkat cell lysates. C) Detection levels of rh-GrM in serum and plasma of healthy donors were strongly decreased.*

synthetic GrM-inhibitor could be of interest. Such inhibitor might lead to a novel therapeutic strategy, in that it can be administrated to patients suffering from an inflammatory disease in which active extracellular GrM is contributing.

Intracellular granzyme inhibitors

The two main functions of intracellular serpins are to protect cells by inhibition of endogenous proteases that leak from vesicles into the cytoplasm or by inhibition of exogenous proteases that enter the cell. In chapter six, we show that SERPINB4 can bind to and inhibit the proteolytic activity of human GrM. SERPINB4 is the first endogenous intracellular inhibitor known for human granzyme M. As SERPINB4 is highly expressed by squamous cell carcinomas and its levels are associated with tumor progression [22,24], our current results suggest that SERPINB4 expression constitutes a novel mechanism by which these tumor cells evade cytotoxic lymphocyte-induced GrM-mediated cell death. The physiological relevance of this hypothesis needs to be investigated. In analogy with SERPINB9 and GrB [25], a target cell line that over-expresses SERPINB4 is expected to be less susceptible to perforin-mediated GrM-induced cell death, as compared to mock transfected cells, implicating a functional

mechanism. We determined an association rate constant for SERPINB4 and GrM of 2.6 x 10^2 $M^{-1}s^{-1}$, which is in the low range of serpin kinetics. This implicates that strong SERPINB4 expression is required to protect target cells against GrM-induced cell death. On the other hand, (hyper)glycosylation of the used purified recombinant GrM protein might negatively affect the kinetic analysis, as discussed in chapter six. When SERPINB4 expression is indeed a mechanism for tumor cells to evade GrM-induced cell death, this knowledge might be used in the clinic for patients with squamous cell carcinomas, a type of tumor that normally over-expresses SERPINB4. SERPINB4 is already widely used as a prognostic marker for these tumors [26]. Together with studies covering the transcriptional regulation of the SERPINB4 gene, our current finding might lead to improved treatment of patients with squamous cell carcinomas in the future. If SERPINB4 expression by tumor cells can be manipulated by drug-intervention, resulting in a decreased SERPINB4 protein expression, these tumors may become more susceptible to GrM-induced cell death, finally resulting in an improved clinical outcome of the patients. On the other hand, identification of stimuli that up-regulate GrM expression by cytotoxic lymphocytes may also result in a beneficial effect for the patients.

Granzymes are highly cytotoxic proteases and therefore their trafficking, storage, and activity in cytotoxic lymphocytes themselves is strictly regulated to prevent unwanted damage [27]. Normally, granzymes are stored in cytotoxic vesicles, of which the content is degranulated into the immunological synapse after recognition of a target cell. However, granzymes can leak from the acidic cytotoxic granules into the pH-neutral cytoplasm of the killer cells, especially following activation and degranulation of cytotoxic lymphocytes, resulting in active granzymes that initiate unwanted suicide pathways. To prevent the onset of cell death routes by misdirected endogenous GrB, cytotoxic lymphocytes express the intracellular cognate inhibitor SERPINB9 [28]. This serpin plays a role in the maintenance of cytotoxic T cell homeostasis and its over-expression enhances the cytotoxic potency of killer cells [29,30]. This concept of expression of endogenous intracellular granzyme inhibitors is also applicable to other cell types. Previously, our group demonstrated that SERPINB9 is expressed by human mast cells [31] and in chapter three we now show granular GrB expression in these cells. So, expression of a protease inhibitor by a specific cell type may indicate that the cognate target protease is also expressed by these cells, and visa versa.

Of the five human granzymes, an endogenous intracellular inhibitor has so far only been known for GrB. In analogy with the concept above, it seems logical that cytotoxic lymphocytes expressing a specific granzyme also harbour the cognate inhibitor in their cytoplasm to secure their viability. For human GrM, we identified SERPINB4 as the first endogenous intracellular inhibitor (chapter six), suggesting that, besides epithelial cells, this serpin may also be expressed by cytotoxic lymphocytes. However, a previous study determined that RNA of SERPINB4 was absent in peripheral blood leukocytes whereas immunohistochemistry neither revealed SERPINB4 protein expression in hematopoietic cells of the bone marrow or lymphoid cells of the lymph node, tonsil, and spleen [32]. Although SERPINB4 expression in cytotoxic lymphocytes requires more investigation, other (non-serpin) inhibitors may protect these cells from misdirected endogenous GrM. On way to identify such a GrM-inhibitor can be via immunoprecipitation of native GrM from a NK cell lysate using one of our novel and specific mAbs against this protease, and subsequent analysis of the proteins that bind to GrM by mass spectrometry. This approach is also applicable to search for endogenous intracellular inhibitors of human GrA, GrH, and GrK, which are unknown so far.

Serpins as tumor markers

Expression of SERPINB8 and SERPINB13 in respectively neuroendocrine tumors of the pancreas (chapter eight) and squamous cell carcinomas of the head and neck region (HNSCC) (chapter seven) has been investigated by immunohistochemistry. Our studies indicate that, for the above mentioned types of cancer, SERPINB8 and SERPINB13 can be used as a diagnostic and prognostic marker, respectively. In the pancreas, SERPINB8 is a useful specific marker to identify the neuroendocrine phenotype of tumors, especially because all tested adenocarcinomas of the pancreas did not express SERPINB8. Addition of SERPINB8 to the general used markers chromogranin A and synaptophysin resulted in a slightly enhanced diagnostic specificity in our panel of islet cell tumors, emphasizing the value of SERPINB8 as marker for these tumors. Investigation of several panels of neuroendocrine tumors from other tissues revealed high expression of SERPINB8 in medullar carcinomas of the thyroid and in carcinoids of the colon as well (unpublished results). However, SERPINB8 was also expressed by exocrine adenocarcinomas of the thyroid and colon, thereby precluding SERPINB8 as a specific marker for neuroendocrine tumors in general.

Down-regulation of SERPINB13 expression in HNSCC positively associated with metastasis of these tumors and poor clinical outcome of the patients (chapter seven). This is the first report concerning the clinical implications of down-regulated SERPINB13 expression in HNSCC and suggests that SERPINB13 may be used as an additional prognostic marker for HNSCC. Similar studies on other HNSCC panels are required to determine whether SERPINB13 indeed serves as a prognostic marker for individual patients, like EGFR and HPV [33]. Future studies that include several of these markers simultaneously can elucidate the additional specificity of SERPINB13 as a prognostic marker for patients with HNSCC.

The expression of serpins in tumors is not generally up- or down-regulated, but is different for the individual serpins. Whereas SERPINB8 was broadly expressed by most pancreatic islet cell tumors (chapter eight), SERPINB13 expression was partially or totally down-regulated in the majority of HNSCC (chapter seven). Previously, it has already been shown that for example SERPINB5 expression is lost in advanced tumors of diverse origin, whereas SERPINB9 is highly expressed in melanomas and lymphomas [34-36]. The expression profile of serpins depends on the type of cancer and, more importantly, the physiological function of each serpin. If a target protease is involved in the onset of cell death pathways, e.g. a granzyme, the corresponding serpin can prevent this and over-expression of this serpin by tumor cells is beneficial for their survival. On the other hand, if a specific protease enhances the motility of tumor cells, down-regulation of the cognate serpin may lead to tumor progression. So far, only few physiological target proteases are known for serpins.

Potential target proteases of SERPINB8 and SERPINB13

The physiological functions of SERPINB8 and SERPINB13 in normal cells are largely unknown, although several target proteases have been identified *in vitro* [37]. Except for the SERPINB8-furin couple [38], the physiological relevance of these serpin-protease combinations remains to be elucidated, especially for SERPINB8, as most identified target proteases reside extracellular. Whereas the expression of SERPINB8 is highly restricted to squamous epithelium, monocytes, and several neuroendocrine cells [39], the prototypical mammalian kexin/subtilisin-like endoproteinase furin is ubiquitinuously expressed [40]. This suggests that, besides furin, SERPINB8 may inhibit even another, yet unidentified, target protease with a similar cellular distribution as SERPINB8. The expression of SERPINB8

in the insulin-producing β-cells (chapter eight) suggests that SERPINB8 inhibits a target protease that is also specifically expressed by these neuroendocrine cells. In addition, the normal tissue distribution of SERPINB13 revealed a high expression by a subset of cells of the pituitary gland (chapter seven). Sequential stainings with diverse hormones indicated that SERPINB13 was expressed by ACTH-producing cells (unpublished results). So, like SERPINB8 [39], SERPINB13 is also expressed by neuroendocrine cells. This may reflect a novel function of these serpins in the proteolytic processing of specific hormones, i.e. insulin and ACTH. Initially, insulin is expressed as a pro-hormone and conversion into fully active mature insulin occurs in secretory granules of β-cells and involves cleavages by two prohormone convertases (PC): PC1/3 and PC2 [41]. These PCs display considerable homology to the SERPINB8 target protease furin; moreover, PC1/3 and PC2 cleave after the same basic consensus sequence Arg-X-Lys/Arg-Arg↓-X as furin [42]. This amino acid sequence is present in the reactive site loop of SERPINB8 [43], which make PC1/3 and PC2 potential targets for inhibition by SERPINB8. However, in neuroendocrine cells, PCs are localized in the trans-Golgi network as well as in secretory vesicles [44,45], whereas SERPINB8 has a cytoplasmic localization [39]. Therefore, direct involvement of SERPINB8 in hormone processing is unlikely, but, like other serpins, SERPINB8 might fulfill a protective function against unwanted proteolytic damage by endogenous granule-leaking PCs. This hypothesis is in line with our finding that most neuroendocrine tumors of the pancreas highly express SERPINB8 (chapter eight), thereby partly securing the viability of these tumor cells.

In vitro studies revealed two potential target proteases of SERPINB13: the lysosomal proteases cathepsin K and L [46,47]. If SERPINB13 protects cells against unwanted proteolytic damage upon leaking of these cathepsins into the cytoplasm, than down-regulation of SERPINB13 in HNSCC is not beneficial for survival of the tumor cells and in contrast with our findings (chapter seven). Therefore, SERPINB13 may inhibit a hereto unknown target protease, or possibly exert an extracellular function. In the latter case, down-regulation of SERPINB13 expression by SCC results in an increased activity of its target protease in the extracellular environment. As cathepsin K and L are known to mediate tumor progression via degradation of extracellular matrix proteins [48,49], a decreased inhibition of these proteases may result in enhanced metastasis of HNSCC. A SERPINB13 knock out mice might decipher the physiological function and target protease of SERPINB13 in normal squamous epithelia and SCC. Such knowledge probably also clarifies if down-regulation of SERPINB13 expression in HNSCC is a cause or consequence of the tumor progression. In the first case, development of specific small synthetic inhibitors against its target protease may lead to a novel therapeutic strategy to treat patients with HNSCC, as natural inhibition then seems to be impaired by the down-regulated expression of SERPINB13.

Conclusions

This thesis provides a better understanding of the expression of certain granzymes and serpins in diverse immune cells and specific types of tumors, respectively. In addition, unique substrates and an intracellular inhibitor of human GrM were identified. This knowledge can result in the application of these proteins as novel diagnostic and/or prognostic markers for specific inflammatory diseases and cancers. Modulation of the protease-inhibitor balance between a granzyme and its cognate inhibitor or a serpin and its target protease might be a future therapeutic strategy to treat inflammatory diseases and cancers. In case of tumors, an increased expression of granzymes by cytotoxic lymphocytes and/or down-regulation

of a specific inhibitor by target cells might result in an increased granzyme activity and subsequently more efficient tumor cell death, which is beneficial for the patient. On the other hand, in inflammatory diseases, enhanced inhibition of the proteolytic activity, either by down-regulation of a protease, up-regulation of the cognate natural inhibitor, or administering of an inhibitory drug, might result in a positive therapeutically effect.

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Chapter 10

Summary
Nederlandse samenvatting
Dankwoord
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Summary

This thesis describes novel insights about the role of granzymes in the immune system, the resistance of tumor cells against the cytotoxic immune response, and the use of serpins as diagnostic and prognostic marker for tumors. These findings may eventually lead to improvement in the diagnosis and treatment of patients with immunological disorders, viral infection, or cancer.

***Chapter 1** provides a general introduction about granzymes and serpins. In addition, it contains the aims and scope of this thesis. **Chapter 2** is a review about all known characteristics of the unique serine protease GrM.*

Granzymes (granule-associated enzymes) belong to the family of serine proteases, can cleave specific substrate-proteins, and in general fulfil a cytotoxic function. Granzymes are specifically expressed by cells of the immune system, especially by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. In these cells, granzymes are stored in granules, together with the pore-forming protein perforin. Upon recognition of a tumor cell or a virus-infected cell, CTL and NK cells secrete these proteins towards the target cell. Perforin facilitates the entry of granzymes into the target cell, where after granzymes can cleave specific intracellular substrates. This results in the onset of certain signalling-cascades, finally leading to cell death of the tumor cell or virus-infected cell. Humans harbour five different granzymes: GrA, GrB, GrH, GrK, and GrM. GrA and GrB have been studied extensively, whereas little is known about the other granzymes. This thesis describes our novel findings regarding the expression and function of GrB and especially GrM.

The balance between proteases and their cognate inhibitors is crucial in many biological processes. **Serpins** (serine protease inhibitors) form the largest family of protease inhibitors and are characterised by a specific mechanism to inhibit target proteases. After a serpin is cleaved in its reactive centre loop by a specific protease, the serpin undergoes a conformational change. This finally results in the formation of a covalent complex between the serpin and the cognate protease, in which they are both inactive. Serpins are involved in many different processes, which depends on their specificity and localisation (extracellular or intracellular). The human family of intracellular serpins (B-clade) consists of thirteen members and primarily protect cells again unwanted proteolytic activity, *e.g.* leakage of lysosomal proteases into the cytoplasm. This thesis describes our novel findings regarding the expression and function of SERPINB4, -B8, and -B13.

To discriminate which immunological cell-populations can initiate cell death via a specific granzyme, it is of importance to investigate the cellular expression and regulation of each granzyme in detail.

Chapter 3 presents the expression of **GrB** by human **mast cells**, which corresponds with our previous finding that this multifunctional cell type also expresses the GrB-inhibitor SERPINB9. Depending of the stimuli, GrB is expressed by mature skin-derived primary mast cells, cord blood-derived mast cells, and the human mast cell lines HMC-1 and LAD 2. In these cells, GrB localises in granules and is secreted upon activation. Perforin is not expressed by the stimulated mast cells, except for the cell line HMC-1. This suggests an additional GrB-mediated perforin-independent function for mast cells. In addition, these

results preclude the use of GrB as an exclusive marker for CTL and NK cells.

Chapter 4 describes the expression and regulation of human **GrM** protein by subpopulations of **CTL and NK cells** in detail. So far, GrM expression has been investigated only twice with some controversial results, whereas its regulation has only been studied at the mRNA-level. Flow cytometry analysis with a novel generated specific monoclonal antibody reveals that GrM is expressed by NK cells, NKT cells, and γδ-T cells. $CD8^+$ T cells also express GrM, especially the differentiated effector cells. The regulation of GrM protein expression by a panel of cytokines is completely different as compared with GrB. These results suggest not only a role for GrM in the innate immune response, but also in the adaptive immune response.

The major function of granzymes is the onset of cell death pathways in tumor and virus-infected cells, via hydrolysis of specific substrates. However, these target cells can express specific protease inhibitors to escape the granzyme-mediated cell death mechanism of the immune system. Little is known about how GrM induces cell death, whereas a physiological human intracellular inhibitor of GrM has not been reported yet.

Chapter 5 represents a study to identify novel **GrM**-substrates. Using a proteomics approach, fourteen novel candidate-substrates were identified, including several cytoskeleton-related proteins. Hereof, **α-tubulin** and **ezrin** are both cleaved during GrM-mediated cell death and are direct substrates of the protease. GrM disturbs the polymerisation function of α-tubulin, resulting in disorganisation of the microtubuli network. This mechanism likely contributes to GrM-induced cell death.

Chapter 6 demonstrates that the intracellular serine protease inhibitor **SERPINB4** can inhibit the proteolytic activity of human **GrM**. SERPINB4 forms a typical serpin-protease SDS-stable complex with human GrM. GrM cleaves SERPINB4 in its reactive center loop after the Leucine at the P1-position, which corresponds with the known specificity of the granzyme. A synthetic substrate has been used to determine the kinetic values of this interaction. In addition, SERPINB4 inhibits the hydrolysis of known macromolecular GrM-substrates in a tumor cell lysate. As SERPINB4 is highly expressed by squamous cell carcinomas, these results suggest that SERPINB4 expression constitutes a novel mechanism by which tumor cells evade cytotoxic lymphocyte-induced GrM-mediated cell death.

The function of several intracellular serpins remains to be elucidated, since their physiological target proteases are unknown. As the function of serpins depends on their cellular expression, it is of importance to study the tissue-distribution of serpins in detail. The expression of serpins is often affected in tumor cells, resulting in a misbalance between a serpin and a cognate protease that may influence tumor progression. Therefore, several serpins are used as a diagnostic or prognostic marker for specific types of tumors.

Chapter 7 describes the protein expression of **SERPINB13** in a large panel of normal tissues as well as 99 **head and neck squamous cell carcinomas** (HNSCC). Immunohistochemistry with a novel specific antibody reveals a high expression of SERPINB13 by squamous epithelium. In HNSCC, SERPINB13 is strongly down-regulated, which is consistent with previous studies. For the first time, however, SERPINB13 expression is associated with histopathological and clinical parameters of the tumors and patients, respectively. These analyses demonstrate that down-regulation of SERPINB13 protein expression significantly associates with a poor differentiation grade of the tumor,

the presence of lymph node metastasis, the presence of loss of heterozygosity of the serpin-cluster at chromosome 18q21.3, and a poor disease-free as well as overall survival of the patients. Thus, down-regulation of SERPINB13 protein expression in HNSCC associates with a poor clinical outcome of the patient. Therefore, SERPINB13 may be used as a prognostic marker for HNSCC and seems to be an important protease inhibitor that is involved in the progression of HNSCC.

Chapter 8 presents the expression of **SERPINB8** in normal neuroendocrine cell types and a panel of 20 **neuroendocrine tumors of the pancreas**. In normal neuroendocrine tissues, the highest expression of SERPINB8 is detected in islands of Langerhans, specifically by the insulin-producing β-cells. SERPINB8 is also highly expressed by neuroendocrine tumors of the pancreas, while exocrine tumors of the pancreas totally lack SERPINB8 expression. In island cell tumors, SERPINB8 demonstrates an equal sensitivity as the widely used neuroendocrine markers chromogranine A and synaptophysin. A combination of the three proteins even results in an increased diagnostic sensitivity. Therefore, SERPINB8 can be used as an additional diagnostic immunohistochemical marker for neuroendocrine tumors of the pancreas.

The expression and function of serpins and granzymes have been studied in cells of the immune system and in tumor cells. The obtained results, possible clinical applications, and future study objectives are further discussed in ***chapter 9****.*

Nederlandse samenvatting

De onderzoeksresultaten zoals beschreven in dit proefschrift hebben betrekking op twee type eiwitten: granzymen en serpins. Een uitgebreide introductie over zowel granzymen als serpins staat beschreven in ***hoofdstuk 1****. Daarnaast staat in dit hoofdstuk een beknopt overzicht van de uitgangsdoelen van dit promotieonderzoek.*

Granzymen (granule-geassocieerde enzymen) behoren tot de familie van serine proteasen, kunnen specifieke substraateiwitten knippen en hebben voornamelijk een cytotoxische functie. Granzymen komen specifiek tot expressie in cellen van het immuunsysteem, met name in cytotoxische T lymfocyten (CTL) en natural killer (NK) cellen. In deze cellen worden de granzymen samen met het porievormend eiwit perforine opgeslagen in granules waarbij de proteasen inactief zijn. Na herkenning van een tumor cel of virusgeïnfecteerde cel worden deze eiwitten uitgescheiden door de CTL en NK cellen richting de te doden cel. Perforine faciliteert de toegang van de granzymen tot de doelwit cel, waarna deze inmiddels geactiveerde proteasen specifieke intracellulaire substraten kunnen knippen. Hierdoor worden bepaalde signaleringsroutes aangezet die uiteindelijk zullen leiden tot celdood van de tumor cel of virusgeïnfecteerde cel. In de mens komen vijf verschillende granzymen voor: GrA, GrB, GrH, GrK en GrM. Er is al veel onderzoek gedaan naar GrA en GrB, terwijl nog niet veel bekend is over de andere drie granzymen. In dit proefschrift staan onze nieuwe bevindingen over de expressie en functie van GrB en met name GrM beschreven. Tevens bevat het een overzicht van alle huidige kennis omtrent het unieke serine protease GrM in **hoofdstuk 2**.

De balans tussen proteasen en hun bijbehorende remmers is van belang bij veel biologische processen. **Serpins** (serine protease remmers) vormen de grootste groep protease remmers en worden gekarakteriseerd door een specifiek mechanisme om proteasen te remmen. Nadat een serpin geknipt wordt in zijn typische 'reactive center loop' door een specifiek protease, ondergaat de serpin een grote conformationele verandering. Uiteindelijk resulteert dit in een covalent complex tussen de serpin en het protease waarin ze beide inactief zijn. Serpins zijn betrokken bij heel verschillende processen en dit is afhankelijk van zowel de specificiteit (welk protease kan geremd worden) als de lokalisatie (extracellulair of intracellulair). De humane familie van intracellulaire serpins (B-klasse) bestaat uit dertien leden en beschermen de cel in eerste instantie tegen ongewenste protease activiteit, bijvoorbeeld in het geval van lekkage van lysosymale enzymen in het cytoplasma. In dit proefschrift staan onze nieuwe bevindingen over de expressie en functie van SERPINB4, -B8 en –B13 beschreven.

Om te onderscheiden welke immunologisch celpopulaties celdood kunnen initiëren middels een specifiek granzym is het van belang om precies te weten in welke cellen ieder granzym tot expressie komt en hoe dit gereguleerd wordt.

Hoofdstuk 3 beschrijft de expressie van **GrB** in humane **mestcellen**, wat aansluit bij onze eerdere bevinding dat de GrB-remmer SERPINB9 ook tot expressie komt in dit multifunctionele celtype. Afhankelijk van de stimulatie komt GrB tot expressie in zowel primaire mestcellen geïsoleerd uit huid en tot mestcel-gedifferentieerde uit navelstrengbloed-afkomstige stamcellen, als ook de humane mestcellijnen HMC-1 en LAD-2. In deze cellen lokaliseert GrB in granulen en wordt bovendien uitgescheiden na activatie. Met uitzondering van de mestcellijn HMC-1 komt perforine niet tot expressie in de geactiveerde mestcellen.

Dit suggereert een additionele GrB-gemedieerde perforine-onafhankelijke functie voor mestcellen. Daarnaast toont dit onderzoek aan dat GrB niet als exclusieve marker voor CTL en NK cellen gebruikt kan worden.

Hoofdstuk 4 betreft een gedetailleerde studie naar de eiwit expressie van humaan **GrM** in subpopulaties van **CTL en NK cellen**, aangezien hiernaar tot nu toe slechts twee studies zijn gedaan met controversiële resultaten. Tevens is de regulatie van GrM expressie in deze celtypen nu voor het eerste bestudeerd op eiwit niveau. Flow cytometrie analyse met een nieuw monoklonaal antilichaam toont aan dat GrM tot expressie komt in NK cellen, NKT cellen en γδ-T cellen. Ook $CD8^+$ T cellen brengen GrM tot expressie, met name in de gedifferentieerde effector cellen. Ten opzichte van GrB is de regulatie van GrM expressie door een panel van cytokines totaal anders. Deze resultaten suggereren dat GrM naast algemene reacties door het aangeboren immuun systeem ook betrokken is bij specifiek ontwikkelde T-cel gemedieerde immuun reacties.

De belangrijkste functie van granzymen is het aanzetten van bepaalde celdood routes in een tumor cel of virusgeïnfecteerde cel middels het hydrolyseren van specifieke substraten. Echter, tumor cellen en virusgeïnfecteerde cellen kunnen specifieke protease remmers tot expressie brengen waardoor ze resistent zijn tegen granzym-gemedieerde celdood en dus kunnen ontsnappen aan het immuunsysteem. Voor humaan GrM is het nog nauwelijks bekend hoe dit granzym celdood induceert en een fysiologische intracellulaire remmer is nog nooit beschreven.

Hoofdstuk 5 is een studie naar nieuwe substraten voor **GrM**. Middels een proteomics screen zijn veertien nieuwe kandidaat-substraten van dit protease geïdentificeerd, waaronder enkele eiwitten die deel uitmaken van het cytoskelet van een cel. Hiervan worden de eiwitten **α-tubuline** en **ezrin** ook daadwerkelijk geknipt tijdens GrM-gemedieerde celdood en zijn beide directe substraten. GrM knipt α-tubuline op meerdere plekken en verstoord daarmee de polymerisatie functie van dit eiwit, resulterend in een afwijkend microtubuli netwerk. Dit mechanisme zou bij kunnen dragen aan GrM-gemedieerde celdood.

Hoofdstuk 6 toont aan dat de intracellulaire serine protease remmer **SERPINB4** de proteolytische activiteit van **GrM** kan remmen. Dit gebeurt via het voor serpins typische inhiberende mechanisme, resulterend in een SDS-stabiel complex tussen de twee eiwitten. GrM knipt SERPINB4 in de 'reactive center loop' na het aminozuur Leucine op de P1-positie, wat overeenkomt met de substraat specificiteit van dit granzym. De kinetische parameters van deze interactie zijn bepaald met behulp van een synthetisch substraat en SERPINB4 blijkt ook in een tumor cellysaat de hydrolyse van bekende macromoleculaire GrM-substraten te remmen. Deze bevindingen suggereren dat SERPINB4 expressie, welke verhoogd is in plaveiselceltumoren, een nieuw mechanisme is waardoor tumor cellen resistent zijn tegen een GrM-gemedieerde aanval van het immuun systeem.

De functie van diverse intracellulaire serpins is nog niet opgehelderd, aangezien de te remmen proteasen in fysiologische condities onbekend zijn. Hiervoor is het van belang om de weefseldistributie van deze serpins in detail te bestuderen, omdat de cellulaire expressie van een protease remmer vaak samenhangt met de functie. Dit geldt ook in tumoren, aangezien hierbij vaak een disbalans is tussen een serpin en bijbehorend protease. Daarnaast worden verschillende serpins ook gebruikt als diagnostische of prognostische marker voor bepaalde type tumoren.

Hoofdstuk 7 beschrijft de expressie van **SERPINB13** in een groot panel normale weefsels en 99 **plaveiselcelcarcinomen van het hoofd/halsgebied** (**HNSCC**). Immunohistochemie met een nieuw specifiek monoklonaal antilichaam tegen SERPINB13 laat zien dat deze serpin hoog tot expressie komt in met name plaveiselepitheel. In plaveiselcelcarcinomen is de expressie van SERPINB13 over het algemeen sterk verlaagd, wat overeenkomt met eerdere studies op dit gebied. De SERPINB13 expressie in HNSCC is nu echter voor het eerst geassocieerd met histopathologische en klinische parameters van de tumoren en patiënten. Hieruit blijkt dat een verlaagde SERPINB13 expressie significant associeert met een slechte differentiatie graad van de tumoren, de aanwezigheid van lymfklier metastasen, aanwezigheid van 'loss of heterozygosity' van het serpin-cluster op chromosoom 18q21.3 en zowel een slechte ziekte-vrije als algehele overleving van de patiënten. Dus een verlaagde expressie van SERPINB13 in HNSCC associeert met een slechte klinische uitkomst voor de patiënt. Hierdoor zou SERPINB13 gebruikt kunnen worden als een prognostische marker voor HNSCC en lijkt het een belangrijke protease remmer die betrokken is bij de progressie van HNSCC.

Hoofdstuk 8 beslaat de expressie van **SERPINB8** in normale neuro-endocriene cellen en een panel van 20 **neuro-endocrine pancreas tumoren**. In normale neuro-endocriene weefsels komt SERPINB8 het hoogst tot expressie in de eilandjes van Langerhans, specifiek in de insuline-producerende β-cellen. Ook in endocriene tumoren van de pancreas komt SERPINB8 hoog tot expressie, terwijl exocriene adenocarcinomen van de pancreas deze protease remmer niet tot expressie brengen. In de eilandceltumoren heeft SERPINB8 een zelfde sensitiviteit als de bekende neuro-endocriene markers chromograniene A en synaptofysiene, terwijl een combinatie van deze drie eiwitten zelfs leidt tot een hogere diagnostische sensitiviteit. Daarom zou SERPINB8 gebruikt kunnen worden als een additionele diagnostische immunohistochemische marker voor neuro-endocriene tumoren van de pancreas.

De resultaten zoals beschreven in dit proefschrift beslaan de expressie en functie van serpins en granzymen in tumor cellen en immunologisch cellen. Er zijn nieuwe inzichten verkregen omtrent de rol van granzymen in het afweersysteem, de resistentie van tumorcellen tegen een cytotoxische aanval van het immuunsysteem en het gebruik van serpins als diagnostische en prognostische marker voor tumoren. Dit zou uiteindelijk kunnen leiden tot verbeteringen in de diagnose en behandeling van patiënten met immunologische afwijkingen, virale infecties of tumoren. De resultaten, mogelijke klinische toepassingen en toekomstige onderzoeksdoelen worden verder bediscussieerd in ***hoofdstuk 9.***

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Curriculum Vitae

Pieter de Koning werd geboren op 29 april 1979 te Sint-Oedenrode. In 1985 verhuisde het gezin naar Lieshout, waar Pieter de rest van zijn jeugd doorbracht. Vanaf 1991 ging hij naar het Atheneum van het Macropedius college (Commanderij college) te Gemert, welk in 1997 succesvol werd afgerond.

In datzelfde jaar begon Pieter met de studie Medische Biologie aan de Universiteit Utrecht. Tijdens het laatste deel van zijn studie liep hij drie wetenschappelijke stages. Eerst bij de afdeling Moleculaire Celbiologie van de Universiteit Utrecht, waar signaleringseiwitten werden bestudeerd in kankercellen onder begeleiding van dr. Jord C. Stam. Vervolgens werd onderzoek gedaan naar mestcellen bij de afdeling Dermatologie van het UMC Utrecht onder begeleiding van dr. Edward F. Knol. Tot slot ging hij naar het Swiss Institute for Allergy and Asthma Research (SIAF) te Davos, Zwitserland, waar celdood van T cellen bij allergische patiënten werd onderzocht in de groep van Prof. dr. Cezmi A. Akdis. Naast zijn studie was hij actief bij de studievereniging M.B.V. Mebiose, waarvoor hij in 2001-2002 bestuurslid (fiscus) was. In oktober 2003 ronde Pieter zijn studie Medische Biologie succesvol af met het doctoraal diploma.

In februari 2004 begon Pieter met zijn promotieonderzoek bij de afdeling Pathologie in het UMC Utrecht, waarvan de resultaten zijn beschreven in dit proefschrift. Gedurende vijf jaar deed hij onderzoek naar de expressie en functie van diverse serpins en granzymen, onder begeleiding van Prof. dr. J. Alain Kummer en dr. Niels Bovenschen. Pieter heeft de resultaten middels voordrachten en posters gepresenteerd op diverse (inter)nationale congressen en het werk is grotendeels gepubliceerd in wetenschappelijke tijdschriften.

Per 1 maart 2009 is Pieter werkzaam als ‘stafmedewerker onderzoek’ bij de divisie Heelkundige Specialismen in het UMC Utrecht.