Granzyme B-induced apoptosis in cancer cells and its regulation (Review)

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Abstract. The granzyme B-induced cell death has been traditionally viewed as a primary mechanism that is used by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells to eliminate harmful target cells including allogeneic, virally infected and tumour cells. Granzyme B (GrB) is the most abundant serine protease which is stored in secretory granules of CTLs and NK cells. After recognition of the target cell, the engaged CTLs and NK cells vectorially secrete GrB along with other granule proteins including perforin into the immunological synapse. From this submicroscopic intercellular cleft GrB translocates into the cytoplasm of the target cell. Although several models have been proposed to explain the GrB delivery mechanism, conclusive understanding of this process remains still elusive. Once in the cytoplasm, GrB cleaves and activates, or inactivates, multiple protein substrates, resulting eventually into apoptotic demise of the target cell. This review is focused on the gene structure and expression of GrB, its biosynthesis and activation, delivery mechanisms into the target cell cytoplasm, direct proteolytic involvement in activation of several pro-apoptotic pathways, and on regulation of its activity in cancer cells. Moreover, emphasis is given to the GrB-mediated anticancer effects and future clinical applications of the GrB-based and tumour-targeted recombinant fusion constructs.

Contents

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1. Introduction

Susceptibility of tumour cells to apoptotic death depends on their capabilities to express the components of apoptosis pathways and to activate them in response to extrinsic or intrinsic death signals. The extrinsic death pathways are represented by the death receptor- and the cytotoxic granule-mediated pathways. On the other hand, the intrinsic death mechanisms are represented by the mitochondrial, lysosomal and PIDDosome death pathways.

The death receptor pathway is triggered by the binding of a death ligand, such as FasL (also known as CD95L) and TRAIL (also known as APO-2L) to a specific transmembrane death receptors, Fas (also known as APO-1/CD95) and death receptor 4 and/or 5 (DR4, DR5), respectively (1,2). After binding of the cytosolic Fas-associated death domain adaptor protein (FADD, also known as MORT1) to the liganded death receptors, the initiator procaspase-8 and/or -10 are bound to engaged FADD completing the formation of the death-inducing signalling complexes (DISCs) (1-4). Within DISCs, procaspase-8 and -10 are activated via homodimerization and the active caspase-8 and -10, arising through interdimer proteolytic processing, dissociate from DISCs into the cytoplasm where they cleave and activate procaspase-3 and -7 (1,2,5,6). In addition, both caspase-8 and -10 also cleave the cytosolic BH3-interacting domain death agonist (Bid) protein (7,8). The C-terminal fragment, t(c)Bid, which is formed binds to mitochondria, induces efflux of mitochondrial holocytochrome-c (cyt-c) and other pro-apoptotic mitochondrial proteins into the cytoplasm (9).

The mitochondrial pathway is launched in response to a variety of death stimuli such as DNA damage, chemotherapeutic agents or ultraviolet (UV) light. In these instances, the pro-apoptotic proteins Bax or Bak mediate mitochondrial outer membrane permeabilization (MOMP) and release several pro-apoptotic intermembrane mitochondrial proteins such as cyt-c, second mitochondria-derived activator of caspases (Smac), high temperature requirement A2 (HtrA2)/

Omi serine protease, apoptosis inducing factor (AIF) and endonuclease-G (Endo-G) (10-12). In the presence of dATP or ATP, cyt-c binds to apoptotic protease-activating factor-1 (Apaf-1) and induces its oligomerization into a large heptameric complex called apoptosome (13-15). The apoptosome recruits and activates procaspase-9 (16-19). The active apoptosome-bound caspase-9 then activates the zymogens of the apoptotic effector caspase-3 and -7 (20-22). Both Smac and HtrA2 neutralize the anti-apoptotic functions of the inhibitor of apoptosis proteins (IAPs) (12,23), whereas AIF and Endo-G, after translocation into the cell nucleus, are involved in DNA fragmentation (10,24).

Activation of procaspase-2, an another apoptotic initiator, proceeds within a specific activation multiprotein complex called PIDDosome (25-27). In response to various cellular stresses, this complex can be inducibly formed in the cytoplasm and/or in the cell nucleus (27,28). The PIDDosome assembled in the cytosol is build of three layers of homo-oligomerized proteins. The proteins are an autoproteolytic carboxyterminal fragment of the p53-induced protein with a death domain (PIDD-CC), the receptor interacting protein (RIP)-associated ICH-1/CED-3 homologous protein with a death domain (RAIDD), and procaspase-2 (26,28,29). The PIDDosome assembled in the nucleus is composed of PIDD-C and/or PIDD-CC, the catalytic subunit of DNA-dependent protein kinase (DNA-PK_{CS}) and procaspase-2 (27). After release from the RAIDD-PIDDosome, the active caspase-2 cleaves the BH3-only protein Bid, leading to MOMP, and other protein substrates including Golgin 160, DNA fragmentation factor subunit A and some cytoskeletal proteins (30,31).

Apoptotic stimuli, such as oxidative stress, tumour necrosis factor (TNF)-α treatment, lysosomotropic agents, sphingosines, etoposide, UV light, FasL or TRAIL have been shown to trigger lysosomal membrane permeability (32-34). The partial release of some cathepsins including B, K, L, and S into the cytoplasm results in proteolytic fragmentation of Bid protein and release of cyt-c from the t(c)Bid-disrupted mitochondria (35-37).

Both the intrinsic and the extrinsic apoptosis pathways converge on the activation of the effector apoptotic procaspase-3, -6, and -7 (22,34,38,39) as well as they lead to MOMP via cleavage of Bid protein and/or the Mcl-1 protein component of the Mcl-1•Bim complex (7,40-44).

The present review is focused on the role of the serine proteinase granzyme B (GrB) in the cytotoxic granule-mediated apoptosis pathway, which is thought to be the most important mechanism for clearance of cells infected with intracellular pathogens, allogeneic cells and tumour cells (45-49). After the cell recognition-based conjugation of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells with their target cells, the cytotoxic secretory granules of CTLs and NK cells are vectorially transported towards the conjugation zone and their content is released into a submicroscopic intercellular cleft called immunological synapse (IS) (50,51). The most abundant components of cytotoxic granules are GrB and the pore-forming protein perforin (PFN). GrB is considered to be a major effector of target cell killing by NK cells (52). In addition, the cytotoxic granules of human CTLs and NK cells contain also other granzymes (A, H, K and M), which may co-deliver alternative death signals that operate independently of procaspases activation and MOMP induction (47,52,53). The precise mechanism of GrB translocation into the target cell is not known. Once in the target cell cytoplasm, GrB can trigger several apoptotic pathways via direct proteolytic activation of their signalling or executioner components and itself cleaves multiple intracellular housekeeping proteins (Fig. 1).

Many topics regarding the intracellular *versus* extracellular role of GrB in immunity and autoimmunity have been recently reviewed (53-58). In the present article, we review the gene structure and expression of GrB, its biosynthesis and activation, delivery mechanisms into the target cell cytoplasm, and engagement in activation of several pro-apoptotic pathways. Special attention is given to the regulation of GrB proteolytic activity in human cancer cells of solid tumours. Furthermore, we also discuss the promising clinical applications of the GrB-based tumour-targeted therapy, alone or in combination with other anti-tumour treatment.

2. Granzyme B gene organization and regulation of expression

The human GrB gene (*GZMB*) was mapped to the 'chymase locus' on chromosome 14q11.2 (59). This locus contains other three functional genes: granzymes H gene (*GZMH*), cathepsin G gene (*CTSG*), and mast cell chymase gene (*CMA1*). *GZMB* gene is located at the 5' end of the cluster, followed by *GZMH, CTSG,* and *CMA1* genes (60). *GZMB* gene is approximately 3.2 kb in length and is composed of five exons and four introns (61). The leader (signal) sequence of GrB preproprotein is encoded by exon I, the amino acid residues forming the catalytic triad, i.e., His^{57}, Asp^{102}, and Ser^{195}, are encoded within the exons II, III and V, respectively (62).

Studies of the *GZMB* promoter revealed that it contains consensus sequences for binding of several transcription factors, including nuclear factor of activated T cells (NFAT), Ikaros and activator protein-1 (AP-1) (63,64). Recently, a novel nuclear factor-κB (NF-κB) binding site, an enhancer element, responsible for activation of *GZMB* gene transcription in NK cells has been identified outside of the *GZMB* gene, approximately 10 kb downstream from its transcription start point (65). Interestingly, interleukin-3 (IL-3) plays a crucial role in inducible GrB expression in human plasmacytoid dendritic cells and the expression of GrB is regulated on the transcriptional level involving Janus kinase 1 (JAK1) and signal transducer and activator of transcription (STAT)3 and STAT5 (66).

GZMB gene polymorphism has been described in a genetic screen of individuals from various racial groups (67). Compared to the wild-type QPY allele of *GZMB* gene encoding GrB preproprotein with Gln^{48}, Pro^{90}, and Tyr^{247} in its sequence, the RAH allele of *GZMB* gene encodes GrB preproprotein containing Arg^{48}, Ala^{90}, and His^{247} (67). The RAH allele occurs at a frequency rate of 25-30% in each of the racial groups studied and it was proven to represent a neutral *GZMB* gene polymorphism (68).

Initially, it was thought that the GrB expression is restricted to lymphoid cells (45,69,70). However, under certain pro-inflammatory conditions, for instance at a particular

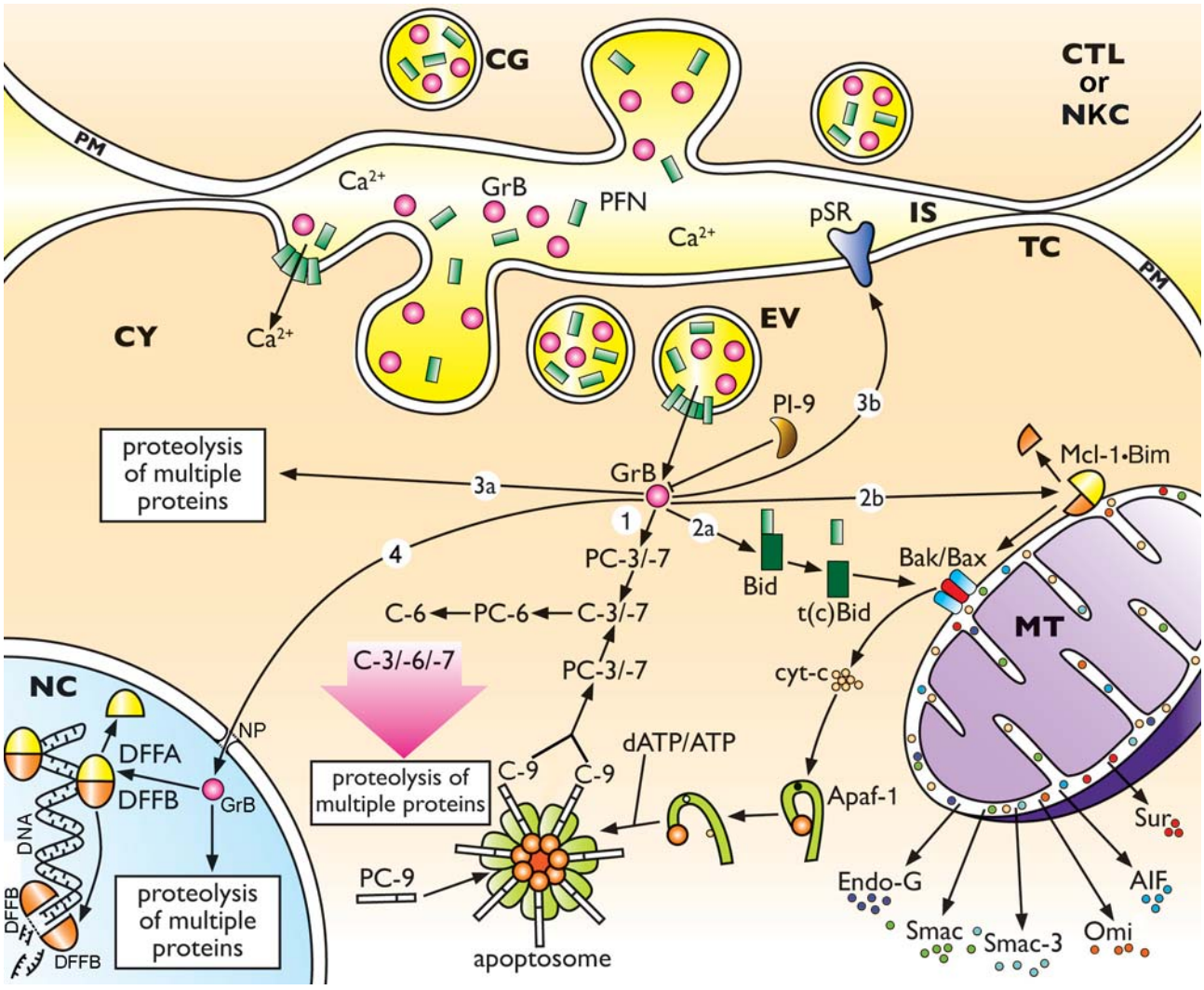

Figure 1. Granzyme B-induced death pathways in cancer cells. [1], Direct proteolytic processing and activation of the executioner procaspase-3 and -7, followed by the caspase-3-mediated activation of the executioner procaspase-6. The active executioner caspases cleave many intracellular proteins. [2], Mitochondrial outer membrane permeabilization (MOMP) via the GrB-mediated proteolytic [2a] conversion and activation of protein Bid to the MOMP-inducing t(c)Bid fragment, and [2b] disruption of the Bim•Mcl-1 complex, involving fragmentation of the Mcl-1 component, and hence derepression of the MOMP-activity of Bim, followed by cytosolic release of several pro-apoptotic proteins (cyt-c, Smac, Smac-3, Omi/HtrA2, AIF and Endo-G) and the inhibitor-of apoptosis protein survivin (Sur). [3a], Proteolytic fragmentation of multiple housekeeping proteins in the cytoplasm (Table I), including [3b] the cytosol-facing signalling domains of some pro-survival plasma membrane receptors (pSR). [4], Translocation into the nucleus and proteolytic fragmentation of multiple intranuclear proteins (Table I), including the DFFA subunit of DNA fragmentation factor (DFFA•DFFB), which leads to the homodimerization-mediated activation of its deoxyribonuclease subunit DFFB. See the text for a more detailed description. CTL, cytotoxic lymphocyte; NKC, natural killer cell; CG, cytotoxic granule; IS, immunological synapse; PM, plasma membrane; TC, target cell; EV, endocytic vesicle; CY, cytoplasm; MT, mitochondrion; NC, nucleus; NP, nuclear pore; PC, procaspase; C, caspase.

composition of the extracellular cytokine milieu, the nature of various receptors engagement, and the presence of regulatory CD4+ T cells, GrB can be expressed not only in normal cells of haematopoietic origin, such as CD4+ T cells, mast cells, activated macrophages and Kupffer cells, neutrophils, basophils and dendritic cells, but also in normal cells of non-haematopoietic origin, including chodrocytes, keratinocytes, type II pneumocytes, Sertoli cells, primary spermatocytes, and cells of granulosa and syncytiotrophoblast (71-87).

Interestingly, GrB has been detected in cancer cells of primary human breast carcinomas (88,89), lung carcinomas (88), urothelial carcinomas (90) and in nasal-type NK/T-cell lymphoma (91). Recently, GrB expression was revealed by immunohistochemistry in oral squamous cell carcinoma and it seems to be localized also in cancer cells (92). Considering these observations, we analysed the expression status of GrB mRNA in non-small cell lung carcinoma (NSCLC) cell lines and NSCLC tumours and matched lungs from surgically treated patients using uncoupled real-time RT-PCR. All examined NSCLC cell lines expressed GrB mRNA but its level was quite low and variable (Fig. 2A). However, compared to NSCLC cell lines, the expression of GrB mRNA in NSCLC tumours was substantially higher (Fig. 2B). In addition, there was no statistically significant difference in the GrB mRNA expression in NSCLC tumours and matched lungs (Fig. 2B) (Krepela *et al*, unpublished data). These results indicate that the lung cancer cells themselves are not the major source of GrB expression in the lung tumours. This notion is further supported by undetectable expression of endogenous GrB protein in NSCLC cell lines (93).

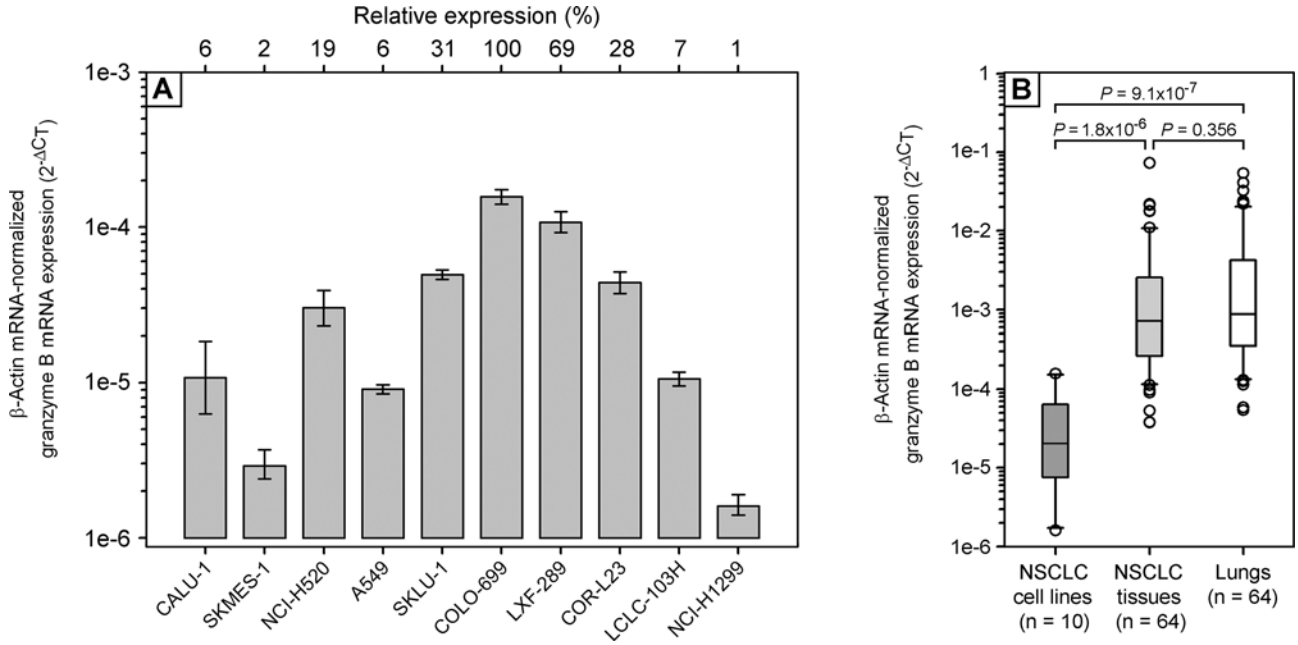

Figure 2. Expression of granzyme B mRNA in non-small cell lung carcinoma (NSCLC) cell lines and NSCLC tissues and lungs as analysed by uncoupled real-time RT-PCR. The sequences of the forward primer, the reverse primer, and the fluorogenic TaqMan probe used for quantification of granzyme B mRNA expression were, respectively: 5'-CTACTGCAGCTGGAGAGAAAGG-3', 5'-CAGCCGGCCACACTGCATGTCT-3', and 5'-(6FAM)GTACTGTCGTAA TAATGGCGTAAGTC(TAMRA)-3'. The sequences of the forward primer, the reverse primer, and the fluorogenic TaqMan probe used for quantification of ß-actin mRNA (an endogenous reference transcript) expression were, respectively: 5'-CTGGCACCCAGCACAATG-3', 5'-GGGCCGGACTCGTCATAC-3', and 5'-(VIC)AGCCGCCGATCCACACGGAGT(TAMRA)-3'. (A), Relative levels of expression of ß-actin mRNA-normalized granzyme B mRNA in NSCLC cell lines. Data indicated as mean ± SEM from three independent experiments. (B), Comparison of the ß-actin mRNA-normalized granzyme B mRNA expression in NSCLC cell lines and NSCLC tissues and matched lungs from surgically treated patients. In the box plot, the upper and the lower boundary of the box and the line within the box indicate the 75th and 25th percentiles and the median, respectively. The error bars above and below the box indicate the 90th and 10th percentiles. Statistical difference (P) between the granzyme B mRNA expression levels was calculated by Mann-Whitney test.

3. Granzyme B biosynthesis, subcellular localization and activation

Human preproGrB mRNA was first identified in 1987 (94) and one year later human GrB cDNA was cloned (95,96). The human GrB protein was first purified and characterized in 1991 (97). GrB and other known human granzymes (A, H, K and M) as well as the closely related myeloid serine proteases, such as cathepsin G, are members of the chymotrypsin superfamily. Like other granzymes, GrB is synthesised as a pre-proenzyme while its signal peptide (i.e., the 18 amino acid residues long N-terminal pre-part), directing the nascent polypeptide chain of the protein into the endoplasmatic reticulum (ER), is removed co-translationally. The resulting proGrB, covalently modified with a mannose-6-phosphate (M6P) group, is transported in ER-derived vesicles to the Golgi apparatus (GA) (62,99). In GA, the M6P moiety serves for sorting out and targeting proGrB and other progranzymes to the GA-derived secretory granules (99). Once inside the secretory granules, proGrB is primarily activated by removal of the N-terminal dipeptide GlyGlu by co-segregated dipeptidyl peptidase I (DPPI; cathepsin C) (62,98,100,101). Failure to remove the GlyGlu propeptide would disrupt the formation of the GrB catalytic site. Besides the major mode of proGrB activation by DPPI, there are other less defined mechanisms of proGrB proteolytic activation (102-104). Recently, the lysosomal cathepsin H was identified as another activator of proGrB (105).

Within the secretory granules, granzymes are stored in association with the chondroitin sulphate containing proteoglycan serglycin (SG) (106-109). Hitherto evidence suggests that the active granzymes are secreted in a macromolecular complex with SG during the target cell killing (106-109). Characterization of interaction between GrB and SG within granules revealed GrB•SG complexes of two distinct molecular sizes. One of them holds approximately 4-8 molecules of GrB, whereas the other one contained as many as 32 molecules of GrB or other granule proteins (108). Storage of GrB in a scaffolded form in the acidic interior of the secretory granules might minimize the proteolytic activity of GrB (108,109). The GrB molecule alone has a high positive surface charge, but when GrB binds to SG its charge may be substantially neutralized. Therefore, the free GrB molecule differs from the SG-bound GrB in that the former might interact with various negatively charged groups exposed on the cell surface, including phospholipid headgroups and those in glycosaminoglycans.

The newly synthesised GrB is heterogeneously glycosylated. The mature enzyme has two potential glycosylation sites at Asn^{51} and Asn^{84}, where N-linked oligosaccharide chains can be attached (110). The process of GrB glycosylation results in generation of both the 32 and 35 kDa glycosylated forms of GrB. The 32 kDa GrB forms contain high mannose oligosaccharide moieties and accumulate in CTLs after T cell receptor (TCR) stimulation (111). Hence, these forms can be stored in the CTLs secretory granules. In contrast, the 35 kDa

GrB forms, which possess only the complex oligosaccharide groups, are not stored in CTLs and instead they are secreted through the constitutive and less specific calcium-independent secretory pathway after TCR activation (111). When a CTL recognizes its target via the TCR, two events occur: the vectorial exocytosis of the secretory granules towards the IS, and the *de novo* biosynthesis of the lytic proteins, including granzymes and PFN.

4. Granzyme B structure and substrate specificity

GrB is a single chain and single domain serine protease. The crystal structure of human GrB was recently determined and provides some rationale for the substrate specificity of GrB (112,113). The GrB structure is folded into two six-stranded ß-barrels, which are connected by three *trans*-domain segments. The regular secondary structure elements include a helical loop between Ala^{56} and Cys^{58}, a helix involving residues from Asp^{165} to Leu^{172}, and a long C-terminal helix from Phe^{234} to Arg^{244}. The peptide bond between Pro^{224} and Pro^{225} is in the *cis* conformation. The *cis*-conformation of these proline residues orients the positively charged Arg^{226} side chain into the S1 subsite (113).

Similar to caspases, GrB has a preference for cleaving peptide bonds immediately adjacent to Asp residues (97). This specificity is due to the structure of the GrB active site, which contains Arg^{226} residue in the S1 subsite. By the combinatorial chemistry approach, the tetrapeptide sequence IEPD has been identified as the preferred P4-P3-P2-P1 recognition motif of GrB for small peptide substrates, although optimal susbstrate recognition may involve features beyond this tetrapeptide sequence (113-116). Recent data indicate that GrB requires an extended substrate sequence, corresponding to the P4-P4' positions, for specific and efficient binding of protein substrates (116).

5. Granzyme B delivery mechanism into the target cell cytoplasm

How granzymes gain entry into the cytosol of target cells during killer cells attack has been subject of several studies in the past, but the effective delivery mechanism during target cell encounter has not been clarified so far. Upon formation of IS, a temporary intercellular conjugation zone, the lytic granules rapidly move and polarize towards the IS where the granzymes and PFN are subsequently released (50,51). The movement of lytic granules within CTLs towards the target cells is directional and depends on an underlying Ca^{2+}-activated microtubule cytoskeleton and other less defined Ca^{2+}-required molecular events (117). Once arrived at the site of secretion, the membrane of secretory granules fuses with the plasma membrane and their content is discharged into a secretory cleft of IS. The IS functions as a conduit for the transportation of lytic granules content and other soluble factors between the CTLs and the target cell (118). It is still broadly debated whether granzymes enter the target cell cytoplasm through PFN pores formed at the post-synaptic plasma membrane or whether both granzymes and PFN are first endocytosed and the granzymes are subsequently released from endosomes within the cytoplasm (107,119-132).

Granzyme B secretion. The exact mechanism of granzyme release from CTLs and NK cells into the extracellular environment remain enigmatic, but it is likely to involve several mechanisms. The lymphokine activated killer (LAK) cells stimulated to undergo granule exocytosis by phorbol myristic acetate and anti-CD2 monoclonal antibodies released GrB as a neutral, high macromolecular weight complex, which possessed pro-apoptotic activity (106). As a single CTL can kill multiple target cells, it is conceivable that free granzymes may leak from the immunological synapse during a CTL degranulation and moving to another target during serial killing (111,133). Extracellular granzymes may also originate from constitutive non-specific secretion that is observed after TCR activation and/or prolonged exposure to interleukin-2 (111,134). TCR triggering induces *de novo* granzyme synthesis, and it is thought that a proportion of newly synthesized GrB is non-specifically secreted through a non-vectorial pathway. This is due to the absence of accessible mannose-6-phosphate in the glycan moiety of the secreted GrB which cannot be targeted to the lytic granules via the mannose-6-phosphate receptor (111). It was recently found that a portion of GrB secreted into the IS can be recovered back into NK cells via clathrin-dependent endocytosis (135).

In the absence of target cell engagement, a proportion of GrB is constitutively secreted by both CTLs and NK cells. In NK cells, the protease is primarily released in an active form through secretory granules, whereas CTLs primarily secrete inactive GrB zymogen, bypassing the granules (133). To date, it is not known whether the secreted proGrB can be proteolytically converted to the active GrB. There is a possibility that an active DPPI and/or cathepsin H, co-secreted by the CTLs or other adjacent cells, might remove the activation GlyGlu dipeptide en bloc. In addition to that, two sequentially acting aminopeptidases might remove the amino acid residues of the N-terminal dipeptide in a step-by-step fashion.

Mechanism of granzyme B internalization into target cells. How granzymes and perforin enter target cells in not fully understood. Several earlier studies provided evidence that GrB can be taken up first into the endosomal compartment of the cell, but that PFN (or other endosomolytic agent) co-entry is necessary for its translocation into the cytoplasm and the cell nucleus (120-123).

It has been demonstrated that GrB binds to the target cell surface in the concentration-dependent and saturable manners and enters the cells via endocytosis (120). This GrB receptor-mediated endocytosis model has been further refined with the identification of the cation-independent mannose-6-phosphate receptor (CI-MPR) as a plasma membrane receptor for GrB (124,129,136). The process of the CI-MPR-mediated GrB internalization is clathrin and dynamin-dependent (124,137). Recently, it has been showed that PFN triggers a wounded plasma membrane-repair response, which is the clathrin and dynamin-dependent endocytosis and which removes PFN and granzymes from the plasma membrane to early endosomes (126,132). Several groups have proposed alternative mechanisms of GrB receptor-mediated cell entry, e.g., via CD44 molecules with known affinity for serglycin (138) or via Hsp70 which can serve as a GrB receptor (139).

Table I. Granzyme B protein substrates.

Protein	Function	Localization	Cleavage sites	Refs.
Acetylcholine receptor ε subunit (AchRε)	Acetylcholine receptor	Plasma membrane	IDID[195]	(266)
Muscarinic acethylcholine receptor 3 (M3R)	Acethylcholine receptor	Plasma membrane	MDQD[330], PSSD[387]	(277)
ß-Actin	Structural protein	Cytosol	ND[a]	(267,268)
Alanyl tRNA synthetase (ARS)	Translation	Cytosol	VAPD[632]	(269)
Bcl-2-associated athanogene 1 (Bag-1L)	Co-chaperone	Nucleus, cytosol	VTRD[125], VVQD[172]	(193)
Bid	Pro-apoptotic sensor	Cytosol	IEAD[75]	(166,270)
CD3	Signal tranducer	Plasma membrane	Many	(274)
Centromere protein B (CENP-B)	Mitosis	Nucleus	VDSD[457]	(269,275)
Centromere protein C (CENP-C)	Mitosis	Nucleus	ND	(269)
DNA-dependent protein kinase catalytic subunit (DNA-PKcs)	DNA repair	Nucleus	VGPD[2698]	(269,276)
DNA ligase IV/XRCC4	DNA repair	Nucleus	SKDD[254]	(284)
Fibrillarin	rRNA processing	Nucleolus	VGPD[184]	(269)
Fibroblast growth factor receptor-1 (FGFR-1)	FGFs receptor	Plasma membrane	ND	(190)
Filamin	Cytoskeletal protein	Cytosol	Many	(186)
Focal adhesion kinase (FAK)	Signal transducer	Cytosol	VSWD[704], DQTD[772]	(285)
α-Fodrin	Cytoskeletal protein	Cytosol	IVTD[1554], AEID[1961]	(187,277)
Glutamate receptor subunit 3 (GluR3B)	Glutamate receptor	Plasma membrane	ISND[388]	(280)
Hip	Chaperone	Cytosol	IEPD[92], INPD[180]	(192,193)
Histidyl tRNA synthetase (HRS/Jo-1)	Translation	Cytosol	LGPD[48]	(269,278)
Hop	Protein folding	Cytosol	LGVD[186]	(191)
Hsp27	Protein folding	Cytosol	VSLD[100]	(193)
Hsp70	Protein folding	Cytosol	INPD[366]	(190)
Hsp90α	Protein folding	Cytosol	Many	(193)
Hsp90ß	Protein folding	Cytosol	Many	(193)
Isoleucyl tRNA synthetase	Translation	Cytosol	VTPD[983]	(269)
Ki-67	Proliferation	Nucleus	VCTD[1481]	(269)
Ku-70	DNA repair	Nucleus	ISSD[79]	(269)
La/SSB	RNA binding	Nucleus	LEED[220]	(269)
Lamin B	Structural protein	Nuclear lamina	VEVD[231]	(189)
Mi-2	DNA methylation, chromatin remodeling	Nucleus	VDPD[1312]	(269,276)
Mcl-1	Inhibition of MOMP[b]	Mitochondrial outer membrane	PAAD[117], EELD[127], TSTD[157]	(41)
Notch1	Delta-like and Serrate-like ligands receptor	Plasma membrane	ND	(190)
Nuclear mitotic apparatus protein 1 (NuMa)	Mitosis	Nucleus	VATD[1705]	(269,276)
Nucleolus organizing region 90 kDa (NOR-90/UBF)	Transcription factor	Nucleolus	ND	(269)
Nucleophosmin B23	rRNA processing	Nucleolus	LAAD[161], VEVD[122]	(271,272)
PMScl/EXOSC10	mRNA degradation	Cytosol	VEQD[252]	(269)
Poly(ADP)ribose polymerase 1 (PARP1)	Ribosylation	Nucleus	VDPD[537]	(269,281)
Postmeiotic segregation 1 (PMS1)	DNA mismatch repair	Nucleus	ISAD[496]	(269)
Postmeiotic segregation 2 (PMS2)	DNA mismatch repair	Nucleus	VEKD[493]	(269)
Procaspase-3	Apoptosis execution	Cytosol	IETD[175]	(151)
Procaspase-7	Apoptosis execution	Cytosol	IQAD[198]	(151,152)
Procaspase-8	Apoptosis initiation	Cytosol	ND	(160,273)
Procaspase-10	Apoptosis initiation	Cytosol	IEAD[372]	(151)
Procaspase-9	Apoptosis initiation	Cytosol	PEPD[315]	(158)
Procaspase-2	Apoptosis initiation	Cytosol, nucleus	ND	(265)

Table I. Continued.

Protein	Function	Localization	Cleavage sites	Refs.
Pyruvate dehydrogenase complex E2 (PDC-E2)	Acetyl-CoA synthesis	Mitochondria	ND	(282)
Rho-associated coiled coil-containing protein kinase 2 (ROCK II)	Plasma membrane blebbing (zeiosis)	Cytosol	$IGLD^{1131}$	(188)
RNA polymerase I (RNA Pol I)	Transcription	Nucleus	$ICPD^{448}$	(269)
RNA polymerase II (RNA Pol II)	Transcription	Nucleus	$ITPD^{370}$	(269)
Signal recognition particle 72 kDa (SRP-72)	Translation	Cytosol	$VTPD^{573}$	(269)
Subunit A of DNA fragmentation factor (DFFA/DFF45)	Chaperone and inhibitor of DFFB (DFF40) deoxyribonuclease	Nucleus, cytosol	$DETD^{117}$, $VTGD^{6}$	(175,279)
Topoisomerase I (Topo-1)	Transcription	Nucleus	$IEAD^{15}$	(269)
α-Tubulin	Microtubule polymerization/aggregation	Cytosol	$VGVD^{438}$	(185,268)
U1 small nuclear ribonucleoprotein 70 kDa (U1-70 kDa)	RNA processing	Nucleus	$LGND^{409}$	(269)
UBF/NOR-90	Nucleolar transcription factor	Nucleolus	$VRPD^{220}$	(269)
Ubiquitin fusion degradation 2 (UFD2)	Ubiquitination	Nucleus	$VDVD^{123}$	(283)

[a]ND, not determined; [b]MOMP, mitochondrial outer membrane permeabilization.

Although native glycosylated GrB binds to CI-MPR (136), cells lacking this receptor are readily killed by glycosylated GrB (124). Therefore, a different model for GrB cell uptake has been propounded. It suggests that selective GrB receptors may not be required and that the positively charged free GrB (having the isoelectric point of approximately 9.5-10) adsorbs to the targets, having negatively charged surface structures, mostly via non-specific electrostatic interactions (128,140). One difficulty with this model is that the high positive charge of GrB is probably neutralized *in vivo* by SG, rendering adsorption to the cell surface much less efficient. It is therefore to posit that GrB exchanges from SG to more negatively charged elements on the cell surface, such as phospholipid headgroups, sulphated lipids, gangliosides, or heparan sulphate proteoglycans, and is subsequently internalized by absorptive pinocytosis (125,127).

Even though the experimental evidence for the involvement of both the receptor-mediated endocytosis and receptor-independent absorptive pinocytosis of GrB entry into cells is strongly convincing, it is probable that these GrB uptake mechanisms can co-exist in the same cell.

The role of perforin in granzyme B delivery into the target cell cytoplasm. GrB itself does not possess a capacity to bind and disrupt lipid membranes (131). However, there is clear evidence that GrB internalization into a target cell depends on PFN (141). PFN is a pore-forming glycoprotein that can bind to phospholipid components of target cell membranes in the presence of Ca^{2+} ions and subsequently oligomerizes to form pores with a diameter of 5-20 nm (142-145). Contrary to progranzymes, which are activated by dipeptidyl peptidase I (DPPI), proteolytic processing of the perforin precursor occurs in the absence of DPPI activity (98). There are two well-founded hypotheses explaining the process of the PFN-assisted GrB entry into the cytoplasm of target cells. First, the pore entry hypothesis states that GrB and other granzymes are primarily translocated from the cell exterior (e.g., IS) into the cytoplasm through repairable plasma membrane pores by diffusion (146). However, recent data indicate that GrB likely does not enter the cytoplasm through PFN pores at the plasma membrane since inhibiting the PFN-activated endocytosis increases the number of PFN pores persisting at the cell surface, but decreases the GrB uptake (132). Nevertheless, PFN oligomerization and transmembrane pore assembly is a prerequisite for the GrB-induced apoptosis (147). Second, the endosome permeabilization/endosomolysis entry hypothesis claims that GrB and other granzymes are delivered into the target cell cytoplasm after endocytosis and via a PFN-mediated disruptive escape from the endosomal compartment (120, 123,126). The precise mechanism of the perforin-assisted GrB cytosolic translocation from this compartment has not been elucidated yet.

6. Death pathways activated by granzyme B in cancer cells

Once GrB is delivered into the cytosol it can proteolytically attack different protein substrates and initiate programmed cell death. To date, more than three hundreds intracellular and extracellular human proteins as potential GrB substrates have been identified (56,58,148). The list of the proteins cleaved by GrB during apoptosis is indicated in Table I. However, only for a few of them the physiological relevance of their cleavage in the process of cell demise is established.

Direct proteolytic activation of executioner procaspases. GrB has a similar preference as caspases for cleaving protein peptide bonds C-terminal to Asp residue (114,115). GrB is capable of direct proteolytic processing and activation of the executioner procaspase-3 and -7 (43,149-156). On the other hand, there are contradictory reports on the direct GrB-mediated procaspase-6 proteolytic activation (43,157,158). Moreover, the apoptotic procaspases including procaspase-8, -10, -9, and -2 were reported to serve as substrates for the active GrB (43,151, 158-162,265). However, it should be emphasized that GrB can proteolytically cleave these initiator procaspases but it cannot activate them. The initiator procaspases are activated exclusively by homodimerization in specific multiprotein activation platforms such as apoptosome, DISC and PIDDosome (1,4,6,19,31).

Mitochondrial outer membrane permeabilization (MOMP). Some authors believe that GrB preferentially triggers apoptosis through an alteration of the outer mitochondrial membrane (OMM) rather than by the direct activation of the executioner caspases (163). GrB can disrupt OMM at least by two distinct mechanisms. First, GrB cleaves and activates the cytosolic BH3-only protein Bid, which C-terminal proteolytic fragment, t(c)Bid, translocates to outer mitochondrial membrane and promotes its permeabilization (164-169). Through the t(c)Bid-mediated MOMP several pro-apoptotic mitochondrial proteins are released into the cytoplasm and trigger caspase-dependent and -independent death pathways (see above). Second, the proteolytic disruption of the Mcl-1•Bim complex at OMM by GrB may constitute an alternative and/or parallel mechanism of the GrB-induced MOMP. Herein, GrB cleaves the Mcl-1 component of the complex relieving the OMM-permeabilizing activity of Bim (40,41). Moreover, it was recently discovered that GrB translocates into the mitochondria of target cells and cleaves the HS-1-associated protein X-1 (Hax-1) (170). The resulting N-terminal Hax-1 fragment is responsible for mitochondrial depolarization.

Intracellular housekeeping protein substrates of granzyme B. GrB has an important role in dismantling the cytoskeleton by cleaving of several its protein components such as α-tubulin (43), filamin (186), ß-fodrin (187) and Rho-associated coiled coil-containing protein kinase 2 (ROCK II) (188).

Besides that, GrB could disrupt the cohesiveness of the nuclear lamina by direct cleaving of lamin B (189). This opens an intriguing possibility that disruption of the nuclear lamina by GrB and other granzymes facilitates their nuclear entry through nuclear pore complexes (189). Once in the nucleus, GrB cleaves several nuclear protein substrates and thus triggers many critical intranuclear molecular processes (Table I).

Another class of GrB substrates represent transmembrane receptors for growth factors, such as Notch1 and fibroblast growth factor receptor-1 (FGFR-1) that transmit pro-survival and pro-proliferative signals from the extracellular environment (190), and members of the heat shock/stress response family (Hsp) including Hsp70, Hsp90, Bag1-L, Hsp70/Hsp-90-organizing protein (Hop), and Hsc70/Hsp70-interacting protein (Hip) (191-193).

The high sequence homology and conserved primary cleavage specificity of human and mouse granzymes has led to widespread and interchangeable use of human and mouse enzymes in experimental conditions, usually without side-by-side comparisons being made (194-196). Human and mouse GrB exhibit substantial difference in their ability to cleave Bid, as well as several other protein substrates, such as DFFA and procaspase-8 (43,195). Conclusions based on mixing human enzymes with mouse protein substrates and vice versa should be always interpreted with caution.

Intranuclear translocation and direct activation of DNA fragmentation factor. Once delivered into the cytoplasm of target cells, GrB is rapidly translocated to the nucleus (171-173). The detailed mechanism by which GrB translocates into and accumulates within the nucleus and nucleolus is still unclear. Both unglycosylated and the high-mannose glycan moiety bearing GrB molecules can be imported into the nucleus, but GrB molecules containing complex glycan moieties are exclude from the nuclear entry (171). The nuclear GrB import is independent of both ATP and GTP, but it seems to be dependent on certain cytosolic factors including importin (IMP)-α (171-174). GrB delivered into the cytoplasm is a target of the cytosolic proteinase inhibitor-9 (PI-9)/serpinB9 which forms with GrB a covalent inhibitory complex (see below). By competing with free GrB for binding to IMP-α, the GrB•PI-9 complex may prevent the nuclear import of active GrB (174).

Within the nucleus, GrB directly cleaves the subunit A of DNA fragmentation factor (DFF) (175), which is a heterodimer of the inhibitor/chaperone subunit A (DFFA) and the nuclease subunit B (DFFB), and is prebound to DNA (10). The DFFB subunit, escaped from the proteolytically fragmented DFFA subunit, becomes catalytically competent via homodimerization and/or oligomerization and cleaves both strands of the genomic DNA, producing mostly blunt-ended DNA fragments showing a typical oligonucleosomal ladder pattern upon electrophoresis (10,176). The GrB-mediated DFF activation can be an alternative way leading to apoptotic DNA fragmentation in cancer cells which are unable to translocate the active caspase-3, a main DFF activator (176), into the nucleus (cf. 177) or carry a loss-of-function mutation of the *CASP3* gene (178). Besides DFF, GrB can cleave several other nuclear proteins, see Table I.

Detachment of cells from extracellular matrix and anoikis. Both newly synthesized GrB zymogen and active GrB are constitutively and non-specifically released from CTLs (133). Once secreted, the active GrB can cleave extracellular matrix components including vitronectin, fibronectin and laminin (179,180). This can induce the detachment-triggered cell death, i.e., anoikis (179-181). In general, modulation of cell adhesion by GrB may have important biological and pathobiological consequences. Due to extracellular matrix remodelling, GrB may contribute to migration of activated leukocytes through tissues. Moreover, the secreted GrB may either inhibit tumourigenesis via inducing anoikis of tumour cells, e.g., in early stages of tumour development, or it may facilitate tumourigenesis through promoting tumour cell spreading, migration and invasion (181-184).

7. Regulation of granzyme B activity

It remains a puzzle how one CTL can kill multiple target cells over longer periods of time without self-destruction and why tumour cells or virus-infected cells became resistant to granule-mediated apoptosis. Several candidate regulators of GrB activity have been identified. First, the serpin proteinase inhibitor-9 (PI-9)/serpinB9 was identified and established as a powerful inhibitor of GrB (197,198). Second, some granzymes co-secreted with GrB, such as GrM and GrH, have a potential to influence the activity of GrB indirectly. They both can promote GrB activity through the direct cleavage and inactivation of protein inhibitors of GrB, the former destroys PI-9 in a variety of target cells (199) whereas the latter attacks L4-100K protein, the adenoviral GrB inhibitor, in the virally infected cells (200). Moreover, certain cell surface-bound and/or secreted proteases, such as cathepsin B, might play a role in controlling the susceptibility of various tumour cells to the CTL-mediated killing via the proteolytic inactivation of PFN (cf. 201).

Viral GrB inhibitors

Cytokine response modifier A (CrmA). CrmA, a cowpox virus-derived 39 kDa serpin protein, through the inhibition of caspase-1 plays an important role in regulating the response associated with the cowpox virus infection (202). Besides this anti-inflammatory activity, CrmA also suppresses the CTL-induced apoptosis of tumour cells via targeting and inhibiting both the GrB- and caspase-8-initiated death pathways (203-206). Due to the inhibition of GrB, a serine proteinase, and some caspases, which are cysteine proteinases, CrmA is classified as a 'cross-class' protein inhibitor of proteinases (207). Interestingly, CrmA and proteinase inhibitor-9 (PI-9)/serpinB9, a member of the ovalbumin serpin family, show extensive structural homology (197), leading to suggestion that PI-9 might also function as a 'cross-class' protein inhibitor of proteinases (see below).

Adenoviral L4-100K protein. Another member of the viral family of GrB inhibitors is the adenovirus assembly protein L4-100K/Ad5-100K, which is involved in the life cycle of human adenovirus type 5 (Ad5), including virus assembly and activation of late viral protein synthesis (208,209). This protein is a substrate for GrB and inhibits the protease through an unclear mechanism involving interactions of L4-100K with both the active site and an exosite in the GrB molecule (209). The inhibitory effect of L4-100K on GrB can be eliminated by GrH which can proteolytically inactivate this adenoviral protein (200).

Non-viral granzyme B inhibitors

Proteinase inhibitor-9 (serpinB9). Human proteinase inhibitor-9 (PI-9)/serpinB9 is a 42-kDa intracellular protein. It is a member of the serpin superfamily, an ovalbumin family serpin (i.e., a clade B serpin) (210,211). Members of this family lack, by definition, a classical secretory signal peptide (211,212). PI-9 is an efficient and highly specific physiological inhibitor of GrB (K_{ass} = $1.7x10^6$ $M^{-1}\cdot s^{-1}$) that protects CTLs themselves, as well as bystander cells from misdirected GrB (198,213,214).

SERPINB9 gene organization and expression, and subcellular localization and function of proteinase inhibitor-9. SERPINB9 gene has been mapped to a gene cluster on the chromosome 6 at p25. Besides the *SERPINB9* gene, the 6p25 region carries also *SERPINB1* gene, encoding monocyte neutrophil elastase inhibitor (MNEI), and *SERPINB6* gene, encoding cytoplasmic proteinase inhibitor-6 (PI-6) (215-219). *SERPINB9* gene consists of seven exons and six introns (219,220). The translation start site resides in exon 2 and the reactive centre (site) loop (RCL), a region of the PI-9 protein which is proteolytically attacked by GrB, is encoded in exon 7 (220).

PI-9 is expressed abundantly in cells that produce high levels of GrB, i.e., in $CD8^+$ T cells and NK cells (198,214,221). The main physiological function of PI-9 is to protect these cells against the misdirected autogenous GrB (214,222). Moreover, PI-9 may also defend bystander cells or antigen-presenting cells likely to be exposed to GrB during an immune response (214,222). Consistent with such a role is the expression of PI-9 in B cells (198), monocytes (223), mast cells (224), endothelial and mesothelial cells (225), smooth muscle cells (226) and dendritic cells (214,227). Cells at immune-privileged sites, including the eye lens capsula, testes, ovary, placenta, and embryonic stem cells, also upregulate the PI-9 expression (221,225,228).

The expression of PI-9 was also detected at highly variable levels in human cancer cells of carcinomas of the breast, cervix, nasopharynx, esophagus, stomach, colon, and lung, and melanomas (93,229-233). The mechanisms responsible *in vivo* for the differential expression of PI-9 in these cancer cells are not known so far.

In a variety of cells, PI-9 protein is expressed in both the cytoplasm and the nucleus (234). This provides the PI-9-expressing cells with efficient protection against the GrB-mediated damage of target proteins inside of these compartments. The exact mechanisms of PI-9 nuclear import is unknown so far. On the other hand, sensitivity of PI-9 nuclear export to leptomycin B points to the involvement of Crm1 protein (234). This is consisted with the presence of a functionally conserved nuclear export signal in PI-9 protein (235).

PI-9 expression can be upregulated or induced in CTLs, a NK cell line YT-N10, endothelial cells, dendritic cells, human hepatocytes, hepatoma cell lines HepG2 and Huh-7, and gastric cancer cells by several cytokines and inflammatory mediators, such as interleukin (IL)-1ß, IL-18, TNF-α, interferon-α and -γ, 12-O-tetradecanoylphorbol-13-acetate and lipopolysaccharide (214,224,225,233,236-238). There is evidence that an activator protein-1 (AP-1) binding site and two nuclear factor (NF)-κB binding sites in the *SERPINB9* gene promoter are involved in the IL-1ß-mediated PI-9 expression (236). Moreover, it has been demonstrated that estradiol-17ß and other estrogens can induce the expression of PI-9 in human hepatocytes and in the estrogen receptor (ER)-α-positive hepatoma cell line HepG2-ER7 (239,240). The estrogen-mediated induction of PI-9 expression in HepG2-ER7 cells and the breast cancer cells MCF-7 protect the tumour cells against the CTL- and NK cell-triggered apoptosis (241-243). The estrogen-triggered and ER-α-mediated induction of *SERPINB9* gene transcription occurs through a unique estrogen responsive unit (ERU) located approximately

200 nucleotides downstream of the transcription start site. The ERU consists of an imperfect palindromic estrogen response element (ERE) being immediately adjacent to a direct repeat containing two consensus ERE half-sites separated by 13 nucleotides (DR13) (244).

In our studies, we detected only a weak or no increase of PI-9 mRNA expression in the IL-1ß-, IL-18-, and estradiol-17ß-treated NSCLC cell lines (Rousalova *et al*, unpublished data). The highly variable PI-9 expression among NSCLC cell lines (93) together with the lack of PI-9 upregulation in NSCLC cells in response to ILs and estradiol-17ß (Rousalova *et al*, unpublished data) indicate that other factors are involved in the regulation of *SERPINB9* gene expression in these cancer cells.

So far, there is evidence that the upregulated expression of PI-9 in cancer cells may contribute to their resistance against the immune mediated killing and thus it may promote tumour growth and progression (232,243). High levels of PI-9 are associated with a poor therapeutic response and prognosis in lymphomas and melanomas (232,245).

Proteinase inhibitor-9 structure and mechanism of inhibition. PI-9 is composed of 376 amino acids and its tertiary structure consists of nine α-helices (denoted A-I) and three ß-sheets (denoted A-C) (210,220). The regions important for protease inhibition are located on ß-sheet A and the reactive centre loop (RCL). The RCL of PI-9 acts as a pseudosubstrate and contains a GrB cleavage site P1-P1' which equals to the residues E^{340}-C^{341} (116). The RCL segment $VVAE^{340}$-CCME represents an extended P4-P4' region important for interaction with GrB, while the P4' residue E^{344} is required for efficient binding of PI-9 and GrB (116). PI-9 is a direct and irreversible GrB inhibitor which reacts with GrB with a stoichiometry of inhibition of 1:1 (198). Proteolytic cleavage of the PI-9 RCL by GrB causes a rapid conformational change in the serpin, resulting in the formation of a stable serpin-proteinase complex. Thus PI-9 represents a suicide substrate for GrB.

Recent findings indicate that PI-9 can inhibit not only the GrB/perforin-mediated death pathway but also the TNF-α-, TRAIL- and FasL-triggered death pathways (246,247). The inhibition of the death ligand-induced cell killing seems to reside in direct interaction of PI-9 with the intermediate active forms of caspase-8 and -10 (247). This observation points to a possibility to classify PI-9 as a 'cross-class' proteinase inhibitor.

Other granzyme B inhibiting serpins. Another member of the intracellular serpin family, termed raPIT5a, was isolated from the rat pituitary gland (248,249). This protein, expressed also in rat tissues other than the pituitary gland (249), showed a high amino acid sequence similarity to the sequence of PI-9 (248) and its incubation with human GrB resulted in the formation of an SDS-stable enzyme-inhibitor complex (248,249).

Recently, Sipione *et al* discovered that a mouse serpin3n, which is expressed and secreted by Sertoli cells, is an inhibitor of mouse and human GrB (250). This serpin also forms an SDS-stable enzyme-inhibitor complex with GrB (250). In addition, the study also indicates that serpin3n can be involved in the extracellular protection of Sertoli cells from harmful GrB-mediated immune reactions.

8. Granzyme B and anticancer therapy

Cancer disease is still primarily treated by surgery, chemotherapy, and radiotherapy in various combinations. Much hope is currently placed in so-called targeted therapies that can be directed rather selectively against cancer cells and that bypass the damage to normal cells of the body. Several targeting agents have been studied to some extend for clinical use, including monoclonal antibodies and antibody derivates, and more recently also non-immunoglobulin scaffold proteins (251). The utilization of these various affinity proteins in the tumour-targeted therapy can affect tumour growth and progression by altering the signal transduction pathways in cancer cells or by delivery of toxins, cytotoxic drugs or radionuclides to cancer cells.

Granzyme B-protein fusion constructs. The unique mechanism of action of GrB-based and tumour-targeted fusion agents may also enable novel effective combinations with other types of therapeutic agents or with other treatment modalities. Several reports have been published in which GrB was similarly used as an effector death inducing domain being fused to certain tumour targeting sequences (252).

The ErbB receptor family has been extensively studied as predictors in tumour targeting, primarily for therapy using monoclonal antagonistic antibodies and specific tyrosine kinase inhibitors (253). Two receptors in the ErbB family, epidermal growth factor receptor (EGFR) and epidermal growth factor receptor 2 (HER2), are overexpressed in various malignancies and are associated with poor prognosis of cancer patients. Therefore, they are interesting therapy targets in solid tumours. Novel chimeric fusion proteins, immunoGrBs, were generated by sequential fusion of an anti-HER2 single-chain antibody (e23sFv), the Pseudomonas exotoxin-A translocation domain, and the active GrB (254,255). Some of these ternary fusion proteins selectively recognized and destroyed HER2-overexpressing tumour cells both *in vitro* and in tumours xenotransplanted into nude mice (254,255). They have a therapeutic potential especially in conditions when the caspase-dependent apoptosis of cancer cells is inhibited. Dalken and co-workers employed human GrB as an effector function in chimeric fusion proteins that contained the EGFR ligand TGF-α or an ErbB2-specific single-chain antibody fragment (scFv) for selective therapeutic targeting of tumour cells (256). Furthermore, a novel vascular-targeting fusion construct was developed for anti-angiogenic tumour therapy, where GrB was fused with a non-heparin-binding isoform of VEGF (257).

In addition, several authors coupled the active GrB with anti-tumour antibodies, e.g., the anti-melanoma antibody scFvMEL (anti-gp240) (258), the Lewis Y-binding antibody dsFv-B3 (259) or the antibody directed against CD64 (260).

Finally, another potential mechanism how to enhance susceptibility of tumour cells to immune-mediated killing is based on the protection of the caspase-dependent arm of the GrB-mediated apoptosis by neutralization of the inhibitor of apoptosis proteins via the second mitochondria-derived activator of caspase (Smac). This goal can be achieved by overexpression of a recombinant pro-Smac fusion protein, containing a GrB-specific cleavage/activation site, in target tumour cells and subsequent exposure to LAK cells (261).

SAGA fusion construct. Caldas and colleagues developed a novel hybrid vector called Survivin and GrB-induced apoptosis (SAGA). This hybrid cDNA construct contained a fusion of the minimum human survivin gene (*BIRC5*) promoter with the coding sequence of the active form of human GrB. The growth inhibitory effect of SAGA *in vitro* was tested on multiple cancer cell lines, including hepatocellular, colorectal, lung, breast, cervical and ovarian carcinomas, leukemias, central nervous system tumours, soft tissue sarcomas, and osteosarcomas. SAGA alone or in a combination with chemotherapeutic agents, such as vincristine or paclitaxel, efficiently inhibited cell growth in all cancer cell lines tested. Furthermore, the tumour specificity of SAGA was demonstrated (262). Based on these observations, SAGA could represent in future a novel alternative treatment option for many human malignancies which overexpress the survivin gene.

9. Conclusion and the future directions of research

Current evidence indicates that GrB is the main effector of CTLs and NK cells in their killing attack on cancer cells. There are multiple protein targets of GrB within cancer cells and the proteinase, after crossing the membrane of endosomes, is distributed into several subcellular compartments including the cytoplasm, the nucleus and mitochondria. Even though our understanding of GrB involvement in cancer cell apoptosis has been substantially advanced during the last years, the list of cell death-related GrB protein substrates is still expanding and the molecular mechanisms of GrB entry into the cytoplasm, nucleus and mitochondria remain to be clarified. The efficiency of GrB-induced apoptosis of cancer cells mainly depends on the amount of GrB which is delivered into their cytoplasm and escapes from inactivation by PI-9. Although the presence of endogenous GrB is detected in cancer cells of some tumours, it is still unknown whether the proteinase is their own active product or the gained one from CTLs and/or NK cells. If some cancer cells were expressing and releasing the secretory forms of active/activatable GrB, the proteinase might be involved either in promoting or suppressing of both tumour growth and progression (see above). The studies mentioned above demonstrate that delivery of an expressable cDNA fusion construct encoding an active form of GrB into tumour cells might be a promising therapeutic tool for cancer treatment.

Several important questions concerning the involvement of GrB and its specific inactivator PI-9 in apoptosis of cancer cells warrant investigation. First, there is a possibility that certain cancer cell-specific signalling pathways may lead to upregulation of GrB and perforin expression in tumour infiltrating CTLs and NK cells and to targeted delivery of these molecules to cancer cells (cf. 263). Second, studies should address the question whether proGrB is released into the IS during vectorial degranulation of cancer cell-conjugated CTLs and NK cells and whether the proenzyme is subsequently internalized into the cancer cells and undergoes activation there. Third, further studies should clarify if the placement of a hypoxia response element sequence into the survivin promoter fused with cDNA sequence encoding active GrB can increase the expression activity and therapeutic effectiveness of such SAGA construct (262) under hypoxic conditions, which are typically present in solid tumours. Fourth, the positive regulation of transcriptional expression of *SERPINB9* gene by estrogens (241-243) deserves systematic investigation in the estrogen-responsive tumours bearing ERα, especially in breast, uterine and ovarian carcinomas. The rationale for these studies is to elucidate whether the overexpression of PI-9 may predict more aggressive and therapeutically resistant tumours (cf. 232). Finally, because of profound variability of PI-9 expression in tumours of the same histopathological type, ranging from very high to barely detectable PI-9 mRNA levels (cf. 93,229), it is important to determine whether the *SERPINB9* gene is a target for epigenetic reprogramming in cancer cells (264).

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