
REVIEW

Serine Proteases in Immune Protection of the Small Intestine

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Abstract—The gastrointestinal tract is subject to a huge antigenic load, which is especially significant in the intestinal lumen. Being the connecting link between the organism and the external environment, the small intestine fulfils not only digestive and transport functions, but also protective ones and acts as a selective barrier for the flow of nutrients. This review considers proteases of the protective system of small intestine cells, their biochemical properties and activation mechanisms, and involvement in biochemical processes responsible for normal functioning and defense reactions of the intestine. Serine proteases of intestinal immunity are multifunctional enzymes making proteolytic attack aimed to immediately exterminate aggressive elements of the intestinal contents (allergens, toxins), to activate (inactivate) zymogens, receptors, and peptide hormones, and to hydrolyze protein precursors and other biologically active factors. Proteases of intestinal immunity control the inflammatory response, proliferation of B-lymphocytes, apoptosis, and secretory and contractive activity of the intestine; they release neurogenic factors, inactivate biologically active substances, and are involved in degradation of the intercellular matrix and in tissue remodeling.

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The small intestine is a central part of the digestive system where digestion and absorption of exogenous food substrates are taken into the body [1]. The large boundary surface of the small intestine is in constant contact with numerous antigens: alimentary, viral, medicamentous, and also with the pathogenic and opportunistic pathogenic intestinal flora. As a connecting link between the organism and the external environment, the small intestine plays not only the digestive and transporting role but also acts as a selective barrier to the flow of nutrients.

Stroma of the mucous membrane of the intestine is a kind of reticular tissue enriched with various cellular elements such as fibroblasts, plasmocytes, macrophages, lymphocytes, eosinophils, mast cells, and undifferentiated cells [1]. The totality of these cells and the intercellular substance of the stroma are a powerful barrier against penetration of microorganisms and antigens of the intestinal content into the organism's internal medium.

The close contact with antigens stimulates the development in the small intestine mucosa of powerful lymphoid tissue producing an immunocompetent system, which is the place for cell type reactions and also for sensitization of lymphocytes with subsequent differentiation into plasma cells synthesizing immunoglobulins. Compared with other parts of the digestive system, the

small intestine stroma is the most enriched with free cells. In the intercellular space, lymphocytes represent 10-39% of all cells of the epithelial layer [2], whereas lymphocyte number in the small intestine mucosa is 3-fold higher than in the blood channel [3]. In the small intestine not only monomers, but also large molecular complexes can be absorbed; therefore, the presence in this organ of the powerful barrier of immunocompetent cells is quite reasonable.

The small intestine mucosa contains two populations of morphologically different lymphocytes: intraepithelial lymphocytes and lymphocytes of the *lamina propria* of the mucosa [4, 5]. In the epithelial tissue the lymphocytes are mainly located in the integumentary epithelium (intraepithelial lymphocytes), especially at the villus base, and more seldom on the crypt bottom. Lymphocytes are usually located immediately on the basal membrane, not reaching the free surface of the epithelium [6]. Intraepithelial lymphocytes are mainly represented by cytotoxic T-lymphocytes; they can interact with food particles through receptors [7] and have the activity of killers to tumor cells and other target cells [8]. Unlike intraepithelial lymphocytes, the stromal lymphocytes possess phagocytic activity [9]. Phagocytosis of absorbed nutrients by different groups of immunocompetent cells has been

observed by electron microscopy [2]. Thus, lymphocytes of the intestinal mucosa immunologically control the antigenic and chemical composition of substances penetrating from the chyme into the organism's internal medium.

Some granulocytes and lymphocytes continually migrate across the epithelial integument. The epithelial layer includes lymphocytes penetrating from the stroma of villi across the basal membrane. Sometimes lymphocytes come into the lumen of the small intestine [1].

There are numerous mast cells in organs and tissues (skin, mucous surfaces of respiratory, digestive, etc. systems) that are in direct contact with the external environment. The functions of mast cells are first of all associated with their involvement in adaptive immunity as effectors of inflammation [10]. Mast cells are present in the small intestine mucosa (in the *lamina propria* and in the submucosa) and normally are 2-3% of the total cell number [11, 12]. During the initial stage of pathological processes, the number of mast cells, neutrophils, and other cells of the protective system sharply increases [13].

Some cells of the protective system, such as mast cell, neutrophils, and T-lymphocytes, produce serine proteases that are essentially responsible for functioning of these cells as effectors in defense reactions. These proteases are now under intensive studies because they are supposed to participate in various pathological processes. The purpose of this review is to systematize the available data on properties and role of proteases in intestinal immunity.

MAST CELL PROTEASES

At present, about 50 serine proteases are known that are present in secretory granules of mast cells of humans and various other animals. The great majority of these proteases are tryptases and chymases that possess, respectively, trypsin-like and chymotrypsin-like activity [14, 15]. Cathepsin G and other proteases with combined trypsin- and chymotrypsin-like specificity are found in mast cells of human and some animals (bovine, sheep, goat) [15-18].

Tryptase- and chymase-positive mast cells are revealed in the mucosa and submucosa of the small intestine of some animals (bovine, sheep) and humans under normal conditions (in the absence of disease) by enzyme histochemical and immunochemical approaches [19-22]. The properties of chymase isolated from the bovine duodenum mucosa have been described [23].

TRYPTASES

Tryptases are trypsin-like proteases that are present in significant quantities in mast cells of humans [24] and other animals (dog, sheep, bovine, rodents) [25-27].

There are soluble (secretory) and membrane-bound forms of tryptases [28], and three soluble forms of human tryptase — α , β , and ε — exist [29, 30]. Tryptase β that has three allelic variants (β I, β II, β III) is best studied. The tryptase types are significantly different. Human α -tryptase is not packed in secretory granules and is secreted by mast cells independently of activation of the cells [14, 28], whereas β -tryptase is present in secretory granules and is released from mast cells in response to receptor-mediated stimulation. In the secretory granules, protease molecules are packed very closely with other biomolecular components of the granules, which suggests the formation of a quasi-crystalline structure [31].

Tryptases display pronounced selectivity to peptide and protein substrates [32]. Substrate-binding regions of α - and β -tryptases have some structural differences, and they are thought to be responsible for the inability of α -tryptase, unlike β -tryptase, to degrade fibrinogen [33].

Soluble tryptases are activated inside the cell and released as active toroidal tetramers consisting of four identical subunits [33] and functioning as physiological proteolytic units. Tetrameric tryptases have on their surface conservative domains, the so-called Pro-, Tyr-, and Trp-domains containing significant numbers of these residues. According to crystallographic data, contacts between the subunits in the tetrameric complex are due to interaction of the Pro- and Tyr-domains [33]. Under physiological conditions, tetramer stabilization is favored by complexing with heparin.

Because active centers of the monomers are turned into the oligomer, the interaction of the protease with protein inhibitors, such as serpins, α 2-macroglobulin, pancreatic inhibitor, etc., is spatially difficult, which imparts the resistance of tetramers to the action of the protein inhibitors. Tryptase monomers (with molecular weight of about 30 kDa) have low proteolytic activity due to structural changes in the region of the substrate-binding center of the enzyme during the dissociation of the complex. The spontaneous decomposition of the tetramer leads to inactivation of tryptase, and this helps to regulate the physiologically required level of protease activity [33].

The membrane-bound tryptase γ does not form tetramers. In the structure of tryptase γ , the Pro- and Tyr-domains are replaced by anchor domains that mediate the interaction of the protease with the cell membrane [34]. It is unclear whether tryptase γ can leave the cell surface and function in the intercellular space.

The physiological role of tryptases is mainly associated with inflammatory reactions, because mast cells are the major effectors of inflammation (Fig. 1). Tryptases play a signaling role activating the family of protease-activated receptors (PAR) of intestinal epitheliocytes that affects intercellular contacts [35]. This is associated with disturbance of the integrity of the epithelial barrier responsible for protection of the mucosa against the pen-

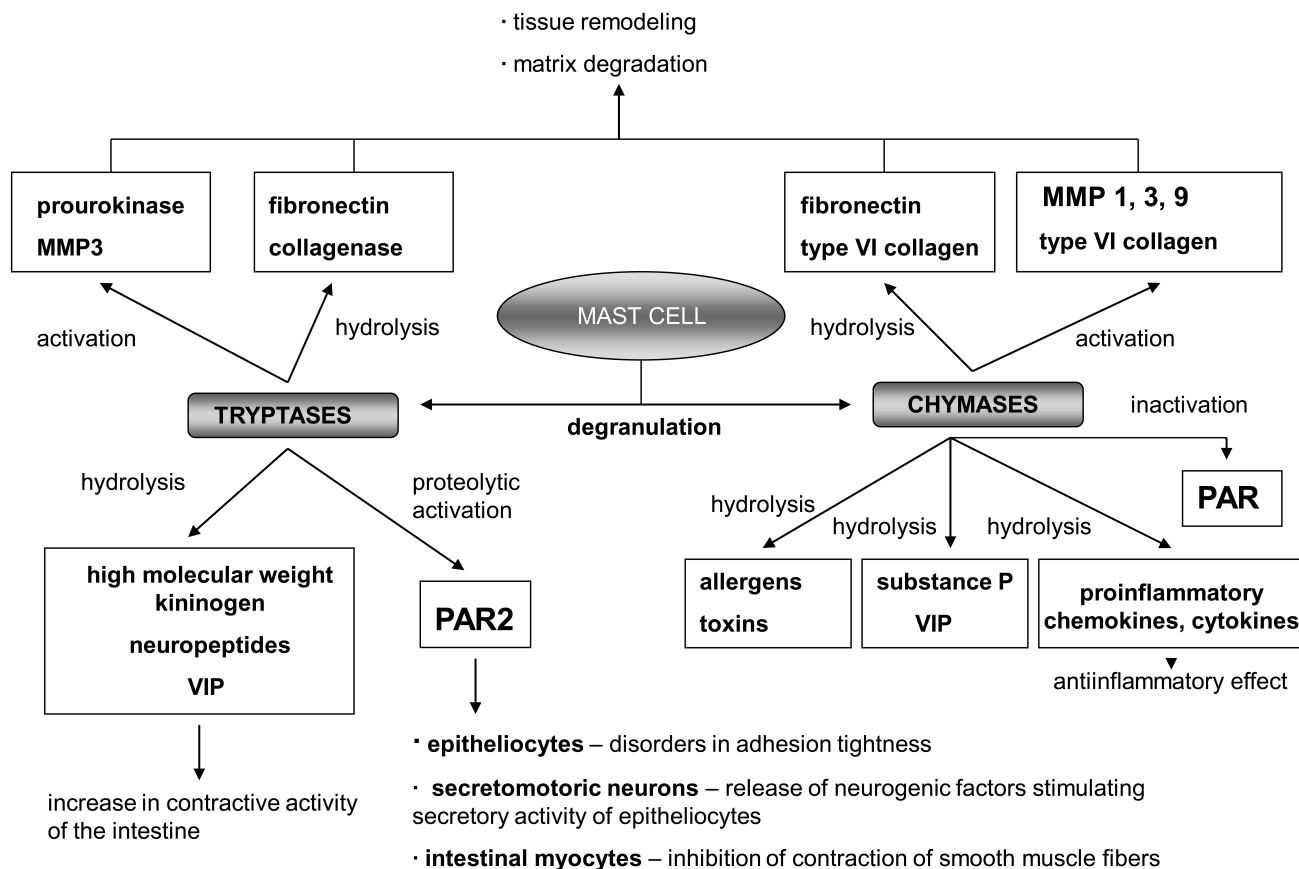


Fig. 1. Biochemical processes in the small intestine controlled by proteases of mast cells. MMP, matrix metalloproteases; PAR, protease-activated receptors.

etration from the intestinal lumen of heterologous macromolecules and microorganisms. Thus, tryptases contribute to increase in the permeability of the intestinal epithelium on inflammatory reactions accompanying various pathological states.

Interaction of mast cells and secretomotoric neurons of the intestine is important for neuroimmune modulation of ion transport in the intestine. Tryptase released during mast cell degranulation activates PAR2 type receptors located on the neuronal surface, which is promoted by nearby locations of mast cells and neurons in the submucosa [36]. In response to PAR2 activation, some neurogenic factors are released that stimulate secretion of electrolytes and fluid by epitheliocytes, and this is an important component of the primary defense of the mucosa against pathogens.

Tryptases can act locally as an anticoagulant, promote an increase in permeability of capillaries, and induce release of neutrophils from lymph into a damage focus [28]. Tryptases are favorable for the contractility of the intestine due to hydrolyzing vasoactive intestinal peptide (VIP), high molecular weight kininogen, and neuropeptides capable of weakening smooth muscles [17, 37]. Concurrently, tryptase inhibits contraction of the intestine

al smooth muscle fibers due to proteolytic activation of the PAR2 type receptor of intestinal monocytes [28].

Mitogenic action of tryptases on epithelial cells [17, 27] provides evidence that these enzymes can be involved in the renovation of the intestinal epithelium. Moreover, tryptases stimulate the release from epitheliocytes of interleukin 8 that, in particular, contributes to regulation of intercellular adhesion molecule-1 expression, and this also confirms the role of tryptases in epithelium renovation.

Human tryptase can directly activate prourokinase and matrix metalloproteinase III, and indirectly – procollagenase [38]. Moreover, tryptase hydrolyzes fibronectin and collagenase IV [39]. These activities of the enzyme indicate its potential involvement in tissue remodeling and degradation of intercellular matrix.

CHYMASES

Mast cells infiltrating the mucosa and in some cases the submucosa of the small intestine of mammals including humans synthesize one or several types of chymases that are monomeric serine proteases with preferentially chymotrypsin-like specificity [14, 21].

Chymases are active at neutral and weakly alkaline pH values characteristic for the physiological medium of the small intestine. The molecular weights of chymases are in the range 24–26 kDa, and some chymases are glycoproteins capable of binding heparin [40]. Chymases are synthesized as inactive preproenzymes containing a hydrophobic signaling peptide and an activating dipeptide with a characteristic sequence Glu-Glu, Gly-Glu, or Gly-Lys. Similarly to tryptases and other proteases of granulocytes, chymases are stored in secretory granules as mature enzyme. The premature manifestation of the protease activity of chymases is prevented by a weakly acidic pH value specific for the contents of secretory granules and the presence of charged proteoglycan [41]. Chymases are presumably processed by dipeptidyl peptidases on early maturation stages of vesicles detached from Golgi complex [42].

The architecture of the substrate-binding site of chymases responsible for their pronounced selectivity to polypeptide substrates is different from that of classic serine proteases [43–45]. Chymases lost in the active site region the disulfide bond specific for classic serine proteases, including tryptases, and this seems to determine the unusual structure of the substrate-binding site of these enzymes [43].

Activities of chymases in the intestine are increased under conditions of pathology associated with hyperplasia of mast cells. The physiological functions of chymases are not fully determined. Based on available data, chymases are supposed to be involved in physiological processes in the small intestine (Fig. 1) via regulation of intestinal hormone processing (hydrolysis of vasoactive intestinal peptide and of substance P) [35], activation of zymogens, degradation of intercellular matrix and tissue remodeling (activation of matrix metalloproteases) [46, 47], direct cleavage of fibers of type VI collagen [44] and of fibronectin [28], and proteolytic degradation of type I procollagen [49]. Chymases can indirectly act on intercellular contacts, which is necessary for migration of mast cells across the epithelium [50], and they regulate the activity of some cells via hydrolysis of PAR type receptors [51]. Chymases possibly display antiinflammatory activity (due to hydrolysis of proinflammatory chemokines and cytokines) [28]. It also seems that chymases can be involved in hydrolysis of heterogeneous peptides and proteins (detoxification and removal of allergens) [52] and in the elimination of intestinal helminths [53], suggesting a possible protective role of these enzymes that is extremely important for the intestine where intervention of heterogeneous elements is highly probable.

CATHEPSIN G

Polymorphonuclear neutrophils are the main source of cathepsin G [54], and this protease is also found in

secretory granules of mast cells [55] and monocytes [56]. Normally, in the small intestine the number of neutrophils in the mucosa is extremely low [57]. In pathologies associated with inflammation, the number of neutrophils in the mucosa sharply increases, the cells migrate across the epithelial barrier, and they appear on the surface of the villi [58]. Neutrophils perform protective functions; in particular, they kill bacteria [59]. In addition to cathepsin G, the secretory granules of polymorphonuclear neutrophils contain other serine proteases (elastase and protease 3) [54]. There are no available data on the presence of these enzymes in the intestinal mucosa, whereas cathepsin G was detected extracellularly in the small intestine of patients with Crohn's disease because of local degranulation of neutrophils [60]. Therefore, the role of cathepsin G in the small intestine seems to be associated with the localization of a pathological process.

Similarly to chymases, cathepsin G is present in cellular granules as active enzyme [61]. Maturation of the preproprotein consisting of 255 residues includes the removal of the signaling peptide of 18 residues, the removal of the *N*-terminal activating dipeptide usual for serine proteases, and *C*-terminal processing [62, 63] resulting in the removal of an 11-member *C*-terminal propeptide. The *N*-terminal processing of cathepsin G is performed by a cysteine dipeptidyl peptidase – cathepsin C [61]. The mature human cathepsin G is a single-chained monomer containing only one glycosylation site [62].

The substrate specificity of human cathepsin G is studied rather well [64, 65]. The enzyme has a combined trypsin- and chymotrypsin-like specificities; it hydrolyzes peptide bonds of the carboxyl group of both positively charged (Lys, Arg) and hydrophobic and aromatic amino acid residues (Phe, Leu, Tyr). Structurally cathepsin G is the most similar to chymases (a conservative set of disulfide bonds including the absence of an S–S-bridge in the active center and characteristic insertions and deletions) [66]. Structural features of the substrate-binding region are favorable for its elevated adaptive properties that allow cathepsin G to interact with various P1-residues [67].

Proteases of neutrophils, including cathepsin G, retain their activity on immobilization on the cell surface [68] due to ionic interaction with involvement of heparin and chondroitin sulfate [69]. The immobilized enzyme is less accessible for the influence of inhibitors.

Cathepsin G cleaves elastin, fibronectin, and collagen, which indicates its possible role in the degradation of the intercellular matrix [54]. Cathepsin G also has bactericidal properties due to presence of a special domain in the enzyme molecule [70].

Cathepsin G can influence signaling mechanisms of cells by processing of chemokines [71] and activation/degradation of receptors [72]. Cathepsin G activates the PAR-2 type receptors of fibroblasts, which results in release of chemokines such as interleukin-8 and protein 1

that are responsible for activation and migration of neutrophils, monocytes, and cytotoxic T-cells into an inflammation focus [73-75]. The PAR-2 type receptors are widely distributed in organs and tissues including the intestine [76]. Available data suggest the leading role of PAR-2 in the regulatory mechanisms of inflammatory reactions [77, 78].

The role of cathepsin G in the intestine is virtually unknown; however, the presence of this protease in certain diseases is established, and this suggests that cathepsin G may be involved in concurrent inflammatory processes.

PROTEASES OF LYMPHOCYTES

The human epithelium contains many lymphocytes — one lymphocyte per 6-10 epitheliocytes). Under normal conditions, epithelial T-lymphocytes can be inactive and display weak cytolytic activity (human) [79, 80] or be active and have pronounced cytolytic activity (mouse) [81]. Under pathological conditions, the number of intestinal T-lymphocytes increases, and they are activated as cytolytic cells. The cell-mediated immune response induces changes in the small intestine epithelium, proliferation of the cryptic epitheliocytes directly depends on the increase in the number of T-lymphocytes in the epithelium, and lymphocytes can be cytotoxic for epitheliocytes [82]. Epithelial lymphocytes probably contribute to pathogenesis of the mucosa [7].

Granules of cytotoxic T-lymphocytes contain many serine proteases, so-called granzymes, which can be released into the intercellular space [83].

Granzyme A. Immunohistochemical study on the human small intestine under normal conditions revealed in the mucosa a significant amount of granzyme A, a trypsin-like protease expressed by non-activated T-lymphocytes. Under normal conditions, about 30% of epithelial lymphocytes of the small intestine mucosa contain granzyme A [84].

Functionally active granzyme A is a 60-kDa homodimer with polypeptide chains connected with a disulfide bond [85]. Processing of the granzyme A zymogen associated with detachment of the activating dipeptide Glu-Arg is catalyzed by cathepsin C (cysteine dipeptidyl peptidase 1); the active enzyme in complex with negatively charged chondroitin sulfate is packed in secretory granules of lymphocytes [86].

In the region of the active center, granzyme A is similar to classic serine proteases such as trypsin, chymotrypsin, plasmin, etc. (presence of the highly conservative disulfide bond Cys191–Cys220 — numeration of chymotrypsinogen). Granzyme A also has the same conservative sequences as chymases and cathepsin G in the *N*-terminal part of the molecule. Moreover, the polypeptide chain of granzyme A has a unique residue Cys76, sta-

bilizing the dimeric form of the enzyme molecule, and a single site of glycosylation [85].

The role of granzyme A seems not to be associated with the cytotoxic activity of T-lymphocytes (Fig. 2) [87]. Granzyme A has trypsin-like activity and can hydrolyze fibrin and other components of the intercellular matrix. Hydrolysis of the intercellular matrix with granzyme A promotes the migration of T- and NK-cells, which are the main effector cells of cellular immunity [88].

Rat granzyme A is an example of an enzyme capable of inducing morphological changes in the epithelial layer of the small intestine and detaching *in vitro* from a support intestinal epitheliocytes immobilized with type IV collagen and fibronectin [89]. The ability of the enzyme to hydrolyze the basal membrane components (type IV collagen, fibronectin) suggests that granzyme A can decrease the adhesion between epithelial cells and the basal membrane [89]. This hypothesis is confirmed by the preferential localization of the enzyme in the upper part of the intestinal villi where mature enterocytes with finished life cycle are desquamated [84, 90].

Granzyme A can induce mitosis of B-lymphocytes, and mouse B-cells are shown to proliferate in the absence of antigen under the influence of this enzyme [83]. Granzyme A induces secretion of cytokines (IL-6, IL-8) by monocytes, which suggests the involvement of the enzyme in inflammatory reactions [91]. The action of granzyme A on the cells is mediated by proteolytic activation of a PAR family thrombin receptor (PAR-1). There are some data indicating antiviral properties of granzyme A [92].

Granzyme B. Granzyme B is present in granules of cytotoxic T-lymphocytes and directly mediates their cytolytic activity [93]. In the normal state of the intestine, granzyme B is expressed poorly [94] because normally the major part of the human intestinal intraepithelial lymphocytes are inactive (see above). The number of granzyme B expressing cells sharply increases in some diseases (celiac disease, chronic inflammation, viral infections) [95]. The maximum cytotoxic activity of T-lymphocytes is preceded by an expression peak of mRNA encoding granzyme B [96]. Thus, under pathological conditions the granzyme B level in the intestinal mucosa increases, and this is associated with an increase in the cytolytic activity of T-lymphocytes.

Granzyme B, a 32-kDa single-chained glycoprotein, has unique substrate specificity among mammalian proteases. Granzyme B hydrolyzes peptide bonds formed by an Asp residue (and by Glu — to a lesser degree) [97]. Granzyme B is structurally similar to protective system cellular proteases such as cathepsin G and chymases. Granzyme B and the above-mentioned proteases have similar positions of disulfide bonds (including the absence of the conservative S–S-bridge in the region of the active center), conservative regions in the *N*-terminal part, and also insertions and deletions in the polypeptide chain that

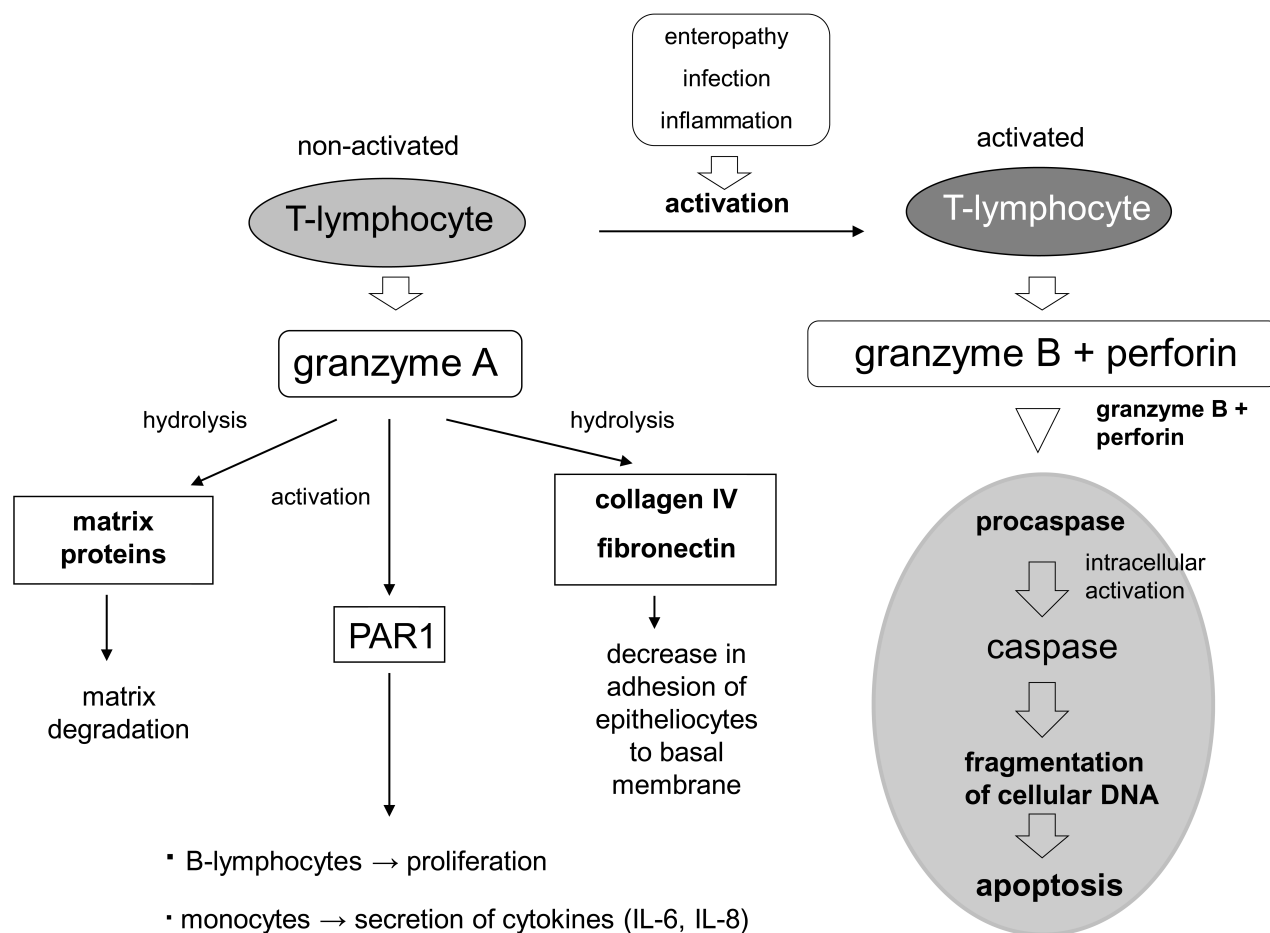


Fig. 2. Granzymes of T-lymphocytes: physiological substrates and role.

discriminate this group from the classic serine proteases [98, 99]. The unique substrate specificity of granzyme B is caused by the presence of the Arg226 residue (numeration by chymotrypsinogen) in the S1-binding center [98]. The processing of granzyme B has the same specific features as processing of related enzymes (intracellular activation of zymogen by cysteine dipeptidyl peptidase before packing into granules) [83].

Granzyme B can induce apoptosis in tumor and virus-infected cells [100]. The penetration of granzyme B into a damaged cell is mediated by the perforin protein of intestinal epitheliocytes. Perforin capable of damaging target cell membrane is released from cytotoxic granules concurrently with granzyme B (Fig. 2) [98]. Granzyme B proteolytically activates a precursor of caspase, which is a key enzyme triggering fragmentation of cellular DNA that finally results in cell death [100]. Granzyme B can participate in caspase-independent cell apoptosis [92]. Some data confirm the involvement of T-lymphocytes containing granzyme B in apoptosis of cryptic cells and in ulcerative damage on inflammatory diseases of the intestine [95].

SERINE PROTEASES IN PROTECTIVE REACTIONS OF THE SMALL INTESTINE

The whole digestive system is especially attacked by pathogens: microorganisms, parasites, allergens, and toxins. Protective reactions in the intestine are significantly associated with the activity of serine proteases synthesized in effector cells of the immune system, such as cytotoxic lymphocytes, mast cells, and neutrophils. Serine proteases of the protective system effector cells are involved in inflammatory reactions and neuroimmune modulation of ion transport, they are responsible for detoxification and elimination of allergens and other heterogeneous substances, and they initiate apoptosis of degenerated and virus-damaged cells, which significantly contributes to the primary defense of the mucosa against pathogens. Serine proteases of immunocompetent cells are most probably multifunctional. These enzymes are supposed to participate not only in the organism's protective reactions but also in the control of normal physiological processes including the contraction of smooth muscles, renovation of epithelium, and tissue remodeling.

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