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Minireview

The role of cystatins in cells of the immune system

Nataša Kopitar-Jerala*

Department of Biochemistry and Molecular Biology, Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia

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Abstract The cystatins constitute a large group of evolutionary related proteins with diverse biological activities. Initially, they were characterized as inhibitors of lysosomal cysteine proteases – cathepsins. Cathepsins are involved in processing and presentation of antigens, as well as several pathological conditions such as inflammation and cancer. Recently, alternative functions of cystatins have been proposed: they also induce tumour necrosis factor and interleukin 10 synthesis and stimulate nitric oxide production. The aim of the present review was the analysis of data on cystatins from NCBI GEO database and the literature, and obtained in microarray and serial analysis of gene expression (SAGE) experiments. The expression of cystatins A, B, C, and F in macrophages, dendritic cells and natural killer cells of the immune system, during differentiation and activation is discussed.

Keywords: Cystatin; Cathepsin; Macrophages; Dendritic cells; Natural killer cells

1. Introduction

Cysteine cathepsins are long known to be responsible for protein degradation in lysosomes. Recent studies show that they are also involved in a number of other important cellular processes such as antigen presentation [1], apoptosis, protein processing [2], as well as several pathologies such as cancer progression [3], inflammation [4] and neurodegeneration [5]. The role of lysosomal cathepsins in antigen presentation has been reviewed recently [6]. Proteinase activity in these processes is highly regulated at the level of protease expression, by regulation of zymogen activation and by expression of endogenous inhibitors. Natural inhibitors that inhibit cysteine cathepsins include cystatins, thyrophins and also some of the serpins [7–9].

*Fax: +386 1 477 39 84.
E-mail address: natasa.kopitar@ijs.si

Abbreviations: BCR, B cell receptor; DC, dendritic cells; FDC, follicular dendritic cells; GC, germinal centres; SAGE, serial analysis of gene expression; MHC, major histocompatibility complex; PKC, protein kinase C; TNF-α, tumour necrosis factor alpha; IL-4, interleukin-4; IL-10, interleukin-10; IFN-γ, interferon-gamma; NK, natural killer cells

The cystatins constitute a large group of evolutionary related proteins acting as protease inhibitors of papain-like cysteine proteases belonging to enzyme family C1 (see the MEROPS database at http://merops.sanger.ac.uk), such as cathepsins B, H, L, and S and legumain-related proteases of the family C13 [10]. Type 1 cystatins, stefins (A and B), are polypeptides of 98 amino acid residues which possess neither disulfide bonds nor carbohydrate side chains and are located mainly intracellularly. Type 2 cystatins C, D, E/M, F, S, SN, and SA are characterized by two conserved disulfide bridges, a larger size (~120 residues) and the presence of a signal peptide for extracellular targeting [11]. Type 3 cystatins, the kininogens, are large (60–120 kDa) multifunctional plasma proteins, containing three type 2 cystatin-like domains containing a total of eight disulfide bridges. Although types 1 and 2 cystatins display considerable differences in amino acid sequence, their tertiary structures are conserved and exhibit a 'cystatin fold' that is formed by five stranded anti-parallel β-pleated sheet wrapped around a five-turn α-helix [12,13]. The structure of human cystatin C in its dimeric form also shows that each one of the two domains in the dimer adopt the typical monomeric 'cystatin fold' [14]. Some type 2 cystatins (C, E/M, and F) are also able to inhibit mammalian legumain, an asparaginyl endopeptidase (AEP), using a binding site distinct from the family C1 interaction site [15]. AEP has been shown to be involved in class II major histocompatibility complex (MHC) restricted antigen presentation [16].

The present review focuses mostly on the expression of two type 1 cystatins: stefins (cystatins) A and B and two type 2 cystatins, cystatins C and F, in cells of the immune system upon differentiation and activation. Two recently developed technologies, oligonucleotide or cDNA microarrays and serial analysis of gene expression (SAGE), allow the determination of the expression patterns of thousands of genes simultaneously [17,18]. The gene expression omnibus (GEO) at the National Center for Biotechnology Information (NCBI) is a large compendium of gene expression data, addressing a wide range of biological issues across many organisms [19]. The aim of the present review is the identification of some of the most interesting questions regarding cystatins in cells of the immune system on the basis of recent data collected in the NCBI GEO and the literature.

2. Stefin A (cystatin A) in follicular dendritic cells (FDC)

Stefin A (cystatin A) has been isolated from epidermis, polymorphonuclear granulocytes, liver, and spleen [20–23]. SAGE

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studies showed that LPS stimulation decreased its synthesis in monocytes [24]. Stefin A was also found in the follicular dendritic cells (FDC) in germinal centres (GC) of human tonsils [25]. In contrast to antigen-presenting cells that present antigens to T cells, FDC do not internalize, process and present antigens in the context of major histocompatibility complex class II (MHC II), but present intact antigen on their cell surface [26,27]. GC form in lymphoid follicles of secondary lymphoid organs and provide an essential microenvironment for T cell-dependent humoral immune responses [28,29]. Within GC, antigen-specific B cells efficiently undergo clonal expansion, isotype switching, somatic mutation, and affinity maturation leading to the generation of plasma and memory cells [30–32]. Only B-cells with the highest affinity B cell receptor (BCR) bind to intact antigens on the surface of FDC and receive survival signals from the FDC whereas low affinity BCR B cells and self-reactive B cell clones are eliminated by apoptosis [31]. Apoptosis in GC B-cells is mainly induced via the death receptor pathway, by the rapid activation of caspase-8 at the level of CD95 death-inducing signalling complex (DISC) [33–35]. GC B cell apoptosis is dependent not only on caspases but also endonucleases [36] and cathepsins [37]. Recently, van Nierop et al. showed that apoptosis in human GC B-cells involved lysosomal destabilization, which was controlled by caspase-8 activity. CD40 ligation provided resistance to lysosomal destabilization, as well as binding of high-affinity B cells to FDC prevented lysosomal leakage and apoptosis in GC B-cells [38]. Van Nierop et al. speculated that besides caspase-8 inhibition there was an additional mechanism to prevent lysosomal instability in adhering GC B-lymphocytes: stefin A, which is expressed at high levels in FDC may play a role in the prevention of apoptosis, as previously proposed by van Eijk et al. [37,39].

3. Cystatins B and C in dendritic cells and macrophages

Cystatin C is the most potent inhibitor of cysteine proteases such as cathepsins B, H, L, and S, with apparent inhibition constants even below the nanomolar range [40]. Mature cystatin C is synthesized as a preprotein with a 26 residue signal peptide. Cystatin C is ubiquitously expressed in all tissues and cell types, although mRNA levels vary several-fold between the tissues [41,42]. Oligomerization of cystatin C leads to amyloid deposits in brain arteries at advanced age but this pathological process is greatly accelerated in the mutant form of cystatin C, responsible for hereditary cystatin C amyloid angiopathy (HCCAA) [43]. Extracellular monomeric cystatin C was found to be internalized by Chinese hamster ovary (CHO) cells and trafficked into lysosomes where it dimerized [44]. Cystatin C-deficient mice have essentially a normal phenotype. Cystatin C-deficient mice showed significantly reduced growth of melanoma lung metastases when compared to wild-type mice [45]. The reason for reduced growth of melanoma lung metastases in cystatin C-deficient mice is unknown, but could be a consequence of an early proteolytic event by a cysteine proteinase during the first hours after administration of melanoma cells [45]. Cystatin C-deficient mice also showed that cystatin C has a protective role in atherogenesis since cystatin C-deficiency promotes atherosclerosis [46]. It was shown that a glycosylated form of cystatin C is a necessary cofactor for fibroblast growth factor 2 (FGF-2) induced mitogenic activity on neural stem cells [47]. Cystatin C *N*-glycosylation was necessary to induce neural stem cell proliferation. The protease inhibitory domain of cystatin C was not directly involved in the process [47]. The unexpected consequences of cystatin C deficiency on the spread of metastasis and atherosclerosis could also be a consequence of alternative functions of cystatin C, possibly as growth factor cofactor.

Dendritic cells (DC) are the professional antigen presenting cells of the immune system. They are defined functionally by their ability to take up antigens such as microorganisms, process them into short antigenic peptides, load the peptides onto major histocompatibility complex (MHC) molecules and then present the resulting complexes at the cell surface. Immature DC are located in the periphery of the body and they take up and process antigens. Activated DC lose their capacity to capture and process antigens. Instead they migrate to the secondary lymphoid organs and present antigen to T cells [48]. Self peptides derived from secretory membrane proteins that are synthesized by the antigen-presenting cells themselves bind to MHC class II molecules tightly, but normally do not activate T cells. Cystatin C peptide (amino acids 40–55) has been found as one of such self peptides bound to MHC class II molecules, indicating that it is endocytosed and cleaved with the antigenic material and then bind to MHC class II molecules [49]. Hashimoto et al. [50] observed that upon DC maturation cystatin C transcripts were significantly downregulated (http://bloodsage.gi.k.u-tokyo.ac.jp/). The SAGE results of Hashimoto et al. were confirmed at the protein level by Zavasnik-Bergant et al. who observed a large increase in intracellular cystatin C during the differentiation of monocytes to immature DC [51], Upon DC maturation, intracellular cystatin C levels decreased and following prolonged incubation of mature DC in the presence of TNF-α, cystatin C was secreted from DC. It has been proposed that cystatin C plays a pivotal role in the control of cleavage and removal of the MHC class II invariant chain (Ii) by regulating the activity of cathepsin S, and hence in the formation of MHC class II-peptide complexes [52]. The work of El Sukkari et al. on DC isolated from cystatin C-deficient mice showed that cystatin C is neither necessary nor sufficient to control MHC class II expression and antigen presentation in DC and that its expression differs between different DC subsets [53]. The absence of cystatin C did not affect the expression, subcellular distribution, or formation of peptide-loaded MHC class II complexes in any of the DC types, nor the efficiency of presentation of exogenous antigens [53]. Recent work by Kitamura et al. showed that interleukin-6 (IL-6)-mediated signalling increased cathepsin S activity, significantly reduced cystatin C expression and reduced the H2-DM and MHC class II αβ dimer levels in DC [54]. Overexpression of cystatin C in DC on the other hand significantly suppressed IL-6-mediated enhancement of cathepsin S activity and reduction of MHC class II αβ dimer, Ii, and H2-DM levels in DC. The authors concluded that the IL-6-mediated alteration of the balance between cystatin C and cathepsin S levels is important for the status of MHC class II αβ dimer, Ii, and H2-DM levels in DC. At least in the system described, cystatin C may regulate cathepsin S activity in immature and mature DC [54]. Murine spleen contains three major endogenous populations of DC. They are referred to as the $CD8^+$ $CD4^-$, $CD8^-$ $CD4^+$, and $CD8^-$ $CD4^-$ subsets [55,56]. $CD8^-$ DC are distinct from $CD8^+$ DC on the basis of a number of criteria and primarily direct a Th2 response by activating T

cells to secrete cytokines such as interleukin-4 (IL-4) [57,58]. $CD8^+$ DC produce IL-12 upon stimulation and induce a Th1 response [59]. Affymetrix microarray gene analysis was used to determine gene expression patterns among murine DC subsets: $CD8^+$ $CD4^-$ and $CD8^-$ $CD4^-$ cells were analyzed directly after sorting and after 2 h cultivation (NCBI GEO GDS352). Cystatin C was upregulated in cultured $CD8^+$DC (NCBI GEO GMS4772, GMS4773), an observation that is in agreement with the study of El-Sukkari et al. [53]. DC also have the capacity to take up, process and present exogenous antigens in association with MHC class I molecules and this pathway is termed cross-presentation [60–62]. $CD8^+$ DC have been shown to be the principal DC subset involved in priming MHC class I-restricted cytotoxic T cell immunity [63]. Since cystatin C is expressed predominately in $CD8^+$DCs, it is possible that it has a role in this process.

Stefin B (cystatin B) is a type 1 cystatin that is distributed rather uniformly among different tissues. In vitro stefin B binds tightly to cathepsins H, L, and S, and less tightly to cathepsin B [9]. Mutations in the gene encoding stefin B are responsible for the primary defect in Unverricht-Lundborg disease (EPM1) [64–66]. Stefin B-deficient mice display a phenotype that is similar to the human disease with progressive ataxia and myoclonic seizures [67]. The mice exhibit apoptosis of cerebellar granullar cells and show increased expression of apoptosis and glial activation genes [68]. It was shown that removal of cathepsin B from cystatin B-deficient mice greatly reduced neuronal apoptosis, but did not rescue animals from ataxia and seizure [69]. Thymocytes from stefin B-deficient mice exerted a markedly increased response when they were exposed to staurosporin, a protein kinase C (PKC) inhibitor compared to thymocytes from wild-type mice [70]. We tested the possibility that stefin B interacts with the receptor for activated PKC (RACK-1) in thymocytes and in this way interferes with PKC signaling in the cells, but the interaction of RACK-1 with stefin B was not confirmed. Preincubation of cells with E-64d did not prevent apoptosis, indicating that staurosporin induces apoptosis in a cathepsin-independent and caspase-dependent manner. Brannvallet et al. reported that stefin B is localized mainly in the nucleus of neural steam cells and in neurons, while in glia cells it is also in the cytoplasm and in the lysosomes [71]. Hashimoto et al. showed that gene transcripts of stefin B were significantly increased upon differentiation of monocytes into macrophages [72]. However, upregulation of the expression of the inhibitor upon differentiation of macrophages does not result in co-localization or interaction with cathepsins L, S or B (Kopitar-Jerala, unpublished observations). The SAGE studies showed that treatment with LPS causes upregulation of stefin B expression in human monocytes, whereas cystatin C is not affected, indicating a possible role of stefin B in innate immune response to bacterial infections [24].

Activated macrophages acquire antimicrobial activities involving reactive oxygen species and reactive nitrogen metabolites. Chicken cystatin, cystatin C, and stefin B have been implicated in nitric oxide (NO) production by interferon-γ-activated mouse peritoneal macrophages [73]. Mouse peritoneal macrophages activated with interferon-gamma (IFN-γ) and then stimulated with IFN-γ plus chicken cystatin generated increased amounts of NO in comparison with macrophages only activated with IFN-γ [73]. The biological effect of cystatins as NO synergistic inducers is not related to inhibition of a cysteine proteinase activity since E-64 did not induce any increase in NO. Increased NO was due to increased inducible NO synthase protein synthesis. Further studies of Verdot et al. suggested that chicken cystatin stimulated the release of TNF-α and interleukin-10 (IL-10) by IFN-γ-activated murine peritoneal macrophages [74]. This observation could be of biological importance as cystatin concentrations necessary to upregulate TNF-α, IL-10 and NO synthesis are in the physiological range, as found in human body fluids. The cystatin C-mediated release of TNF-α is probably responsible for the increase in NO production by IFN-γ-activated murine peritoneal macrophages. The findings by Verdot et al. [74] point at a new relationship between cystatins, cytokines, inflammation and immune responses.

In vitro experiments in cell culture models described above were confirmed by experiments in *Leishmania donovani* infected mice [75]. *L. donovani*, the etiological agent for the severe visceral form of leishmaniasis, multiplies in the phagolysosomes of macrophages of the infected host. Treatment of *L. donovani*-infected murine peritoneal macrophages with a combination of chicken cystatin and IFN-γ induced increased production of NO and did overcome the inhibition of NO synthesis driven by *L. donovani* parasites. Mice treated with chicken cystatin and IFN-γ showed reduced splenomegaly, a lowered parasite burden in the spleen and increased production of NO [75]. The infected mice treated with chicken cystatin and IFN-γ were cured by the induction of NO that killed the parasite and the switched $CD4^+$ T cell-mediated immune responses from disease-promoting Th2 cells to the protective Th1 response shown by the increased production of IL-12 and decreased production of IL-4.

4. Cystatin F in NK cells

Cystatin F is expressed in a variety of tissues. Expression is particularly high in the cells and tissues of the immune system: thymus and spleen, monocytes, DC, T-cells and NK cells [76]. Mature cystatin F is composed of 126 amino acid residues. It is synthesized as a preprotein with a 19 residue signal peptide and possesses a unique extension of six amino acids at its N-terminus. In addition to the two disulfide bridges common to all type 2 cystatins, mature cystatin F has two cysteine residues that, form an interinolecular disulfide bridge, as revealed in the crystal structure of the cystatin F dimer [77]. Cappello et al. observred that in U937 cells cystatin F was secreted as a disulfide bridge-linked dimer [78]. Cystatin F dimer is inactive as an inhibitor of papain like cathepsins and can be activated by chemical reduction [79]. As compared to other cystatins, the protein exhibits a distinct inhibitory profile. It binds tightly to cathepsins F, K, L, and V, less tightly to cathepsins S and H, and does not inhibit cathepsins B, C, and X [79]. Cystatin F can inhibit AEP, but with lower affinity as cystatin E/M and C [80]. The recently elucidated crystal structure of cystatin F revealed that two N-linked glycosylation sites of cystatin F may modulate its inhibitory properties, in particular its reduced affinity towards AEP as compared to other cystatins [77].

Cystatin F has also been shown to be strongly upregulated in LPS-stimulated monocyte-derived DC [50]. In the U937 premyeloid cell line, both differentiation towards a granulocytic pathway by all-*trans*-retinoic acid (ATRA) or towards

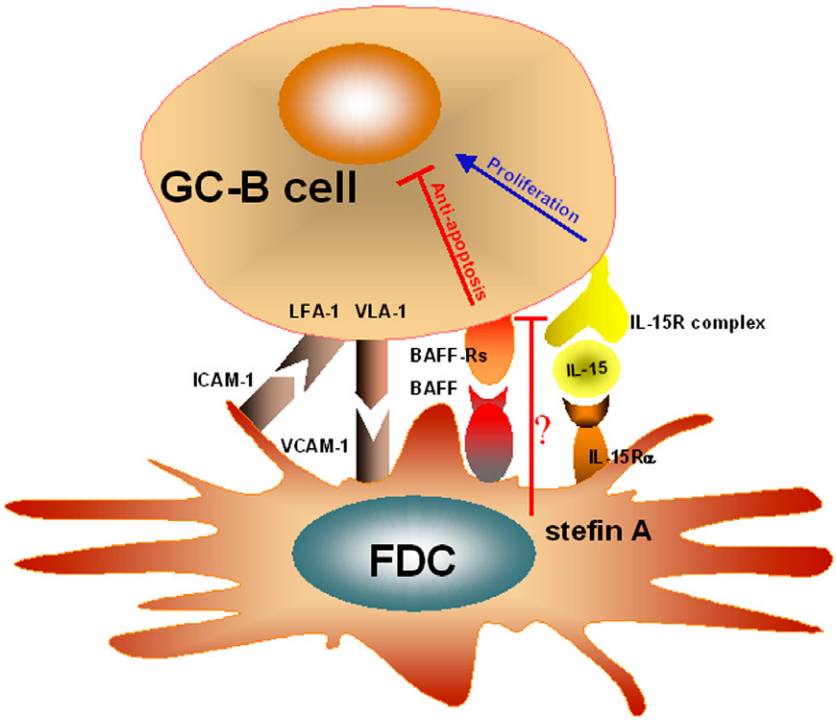

Fig. 1. Stefin A (cystatin A) is expressed at high levels in FDC and may play a role in the prevention of apoptosis in GC B cells as proposed by van Eijk et al. [37,39]. Several signalling molecules are involved in FDC-GC B cell contacts: intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) enhance cell-cell contact; B-cell-activating factor of the tumour necrosis factor family (BAFF/BLys) prevents apoptosis of GC B cells and interleukin-15 (IL-15) stimulate GC B-cell proliferation [28].

a monocytic pathway by stimulation with phorbol ester (TPA) resulted in marked downregulation of cystatin F expression [81].

In U937 cells, cystatin F has been found to be secreted and localized intracellularly in lysosome-like granules [81]. Cappello et al. found it in lysosomes in transfected HeLa cells, but not in U937 cells. Sorting of cystatin F to lysosomes was greatly enhanced when its C-terminal end was extended by few amino acids [78]. The authors concluded that under particular conditions, cystatin F can be sorted to the endocytic pathway, but its unusual inhibitory function is mainly performed extracellularly and probably controlled through dimerization [78]. Langerholc et al. showed that in U937 cells, cystatin F was co-localized with lysosomal markers LAMP-2 and CD68 and when subcellular localization of cystatin F was compared to that of cathepsins, cystatin F was found to be co-localized with cathepsins X and H, but not with cathepsins L, B, C [79]. Further investigations on cystatin F localization, possibly in other cells and in cystatin F-deficient mice, will be necessary to elucidate its biological function (see Fig. 1).

Gene expression analysis of human NK cells and $CD8^+$ T lymphocytes revealed that transcripts of cystatin F were significantly upregulated in NK cells when compared to $CD8^+$T lymphocytes (http://bloodsage.gi.k.u-tokvo.ac.ip/) [82].

NK cells are innate immune lymphocytes that mediate two major functions: recognition and lysis of cancer cells and virus-infected cells and production of immunoregulatory cytokines [83,84]. The activation of NK cells is controlled by complex interactions between activating and inhibitory receptor signals and can be modulated by cytokines [85]. Human NK cells comprise approximately 15% of peripheral blood lymphocytes and the majority of human NK cells are $CD56^{dim}$, whereas a minority are $CD56^{bright}$ and $CD16^{dim/neg}$ [84]. The function of $CD56^{bright}$ NK cells is different from that of $CD56^{dim}$ NK cells. $CD56^{bright}$ NK cells can produce cytokines more abundantly, consistent with their functional role as an innate immunoregulator [86]. In contrast, $CD56^{dim}CD16^+$ NK cells seem to be skewed toward homing to inflammation sites and promoting immune responses, in addition to induction of cytotoxicity [86].

Hanna and coworkers reported that cystatin F transcripts were more abundant in $CD56^{dim}CD16^+$ NK cells and in vitro activated $CD56^+CD16^+$ NK cells than in $CD56^{bright}CD16^-$ NK cells [87] (NCBI GEO GSM26200-5). Although the microarray data do not give us any information about inhibitory activity of cystatin F and have to be interpreted with caution, it is tempting to speculate that cystatin F plays a specific role in the function of NK ($CD56^{dim}CD16^+$) cells.

5. Conclusions

Several structural and kinetic studies have given us insight into interactions of cystatins and cysteine cathepsins in vitro but in vivo very few interactions have been found. The present review aims to identify the most interesting questions rather than providing the definitive answers. The central question remains what the targets of cystatins are that are differentially regulated in cells of the immune system. Is it possible that cystatins have different roles in different tissues like serpins? For example, tPA is not just a 'plasminogen activator'; it is now

widely appreciated for its role in the central nervous system [88,89]. Although it can act on its classical substrate, plasminogen, it also associates with other targets, and in some cases can even act like a cytokine to activate microglial cells without engaging its catalytic properties [90].

The real challenge that lies in front of us is to discover proteinases (and possibly some other proteins) which interact with cystatins that are differentially upregulated in cells of the immune system.

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