Abstract

Cystatins comprise a large superfamily of related proteins with diverse biological activities. They were initially characterised as inhibitors of lysosomal cysteine proteases, however, in recent years some alternative functions for cystatins have been proposed. Cystatins possessing inhibitory function are members of three families, family I (stefins), family II (cystatins) and family III (kininogens). Stefin A is often linked to neoplastic changes in epithelium while another family I cystatin, stefin B is supposed to have a specific role in neurodegenerative diseases. Cystatin C, a typical type II cystatin, is expressed in a variety of human tissues and cells. On the other hand, expression of other type II cystatins is more specific. Cystatin F is an endo/lysosome targeted protease inhibitor, selectively expressed in immune cells, suggesting its role in processes related to immune response. Our recent work points on its role in regulation of dendritic cell maturation and in natural killer cells functional inactivation that may enhance tumor survival. Cystatin E/M expression is mainly restricted to the epithelia of the skin which emphasizes its prominent role in cutaneous biology. Here, we review the current knowledge on type I (stefins A and B) and type II cystatins (cystatins C, F and E/M) in pathologies, with particular emphasis on their suppressive vs. promotional function in the tumorigenesis and metastasis. We proposed that an imbalance between cathepsins and cystatins may attenuate immune cell functions and facilitate tumor cell invasion.

Key words: cystatin; stefin; cathepsin; inhibitor; protease; proteolytic activity; immune cells; tumor; disease.

Introduction

Lysosomal cysteine proteases, the cathepsins, classified as clan C1,¹ were long believed to be responsible for the terminal protein degradation in the lysosomes. This view has changed since they are involved in a number of important cellular processes, such as antigen presentation,² bone resorption,³ apoptosis⁴ and protein processing,⁵ as well as several pathologies such as cancer progression,⁶ inflammation⁷ and neurodegeneration.⁸ So far, cathepsin D, an aspartic protease, and cysteine cathepsins B, H, L, S and few others were associated with cancer progression.⁹⁻¹² Cysteine cathepsins are single-gene products, but the protein products may be polymorphic, due to allelic variants of the gene, alternative RNA splicing and/or post-translational modifications. Similar to other proteases, cathepsins are regulated at every level of their biosynthesis, in particular, by their compartmentalization to lysosomes, activation of pro-enzyme forms and ultimately by their endogenous protein inhibitors.⁴,¹³,¹⁴ Among them the best characterized are cystatins, which comprise a superfamily of evolutionary related proteins, each consisting of at least one domain of 100-120 amino acid res-
idues with conserved sequence motifs. Cystatins function as reversible, tight-binding inhibitors of cysteine proteases, and generally do not possess specific inhibitory activity to particular cathepsin. Type I cystatins (the stefins), stefins A and B, are cytosolic proteins, lacking disulphide bridges. Type II cystatins are more numerous, comprising at least 14 members (for recent review, see Keppler et al., 15). They are extracellular proteins containing two disulphide bridges. Besides well-known cystatins C, D, E/M, F, S, SA, SN this group contains the male reproductive tract cystatins 8 (CRES, cystatin-related epididymal spermatogenic protein), 9 (testatin), 11 and 12 (cystatin T), the bone marrow-derived cystatin-like molecule CLM (cystatin 13) and the secreted phosphoprotein ssp24 (cystatin 14). Type III cystatins, the kininogens, are large multifunctional plasma proteins, containing three type II cystatin-like domains. Another two types of cystatins, fetuins and latexins are constituted by two tandem cystatin domains, however, they do not exhibit inhibitory activity against cathepsins, and are not the subject of the present review.

A broad spectrum of biological roles have been suggested for cystatins, including a role in protein catabolism, regulation of hormone processing and bone resorption, inflammation, antigen presentation and T-cell dependent immune response as well as resistance to various bacterial and viral infections. Cystatins have been suggested as modulators of the proteolytic system in several diseases, including immune disorders and cancer. To highlight the function of cystatins in regulation of proteolysis as well as their functions other than protease inhibition, we review recent finding on the status of type I (stefins A, B) and type II cystatins (cystatins C, F and E/M) in pathological conditions, including their suppressive vs. promotional function in the tumor immunology.

Type I Cystatins

Stefin A (also named cystatin A, acid cysteine protease inhibitor, epidermal SH-protease inhibitor) and stefin B (also named cystatin B, neutral cysteine protease inhibitor) are representatives of family I cystatins. Stefins A and B exhibit 54% sequence identity, both are a 98-amino acid protein with a molecular mass of 11,175 Da and 11,006 Da, respectively. The first three-dimensional structure of a stefin was the crystal structure of the recombinant stefin B in complex with papain. The stefin molecule consists of a five stranded antiparallel β-sheet wrapped around a five turn α-helix with an additional carboxyl-terminal strand that runs along the convex side of the sheet. The N-terminus and the two β-hairpins form the edge of the wedge shaped surface, which bind into the active site cleft of cysteine proteases. The three-dimensional structure of stefin A has been determined in solution and in complex with cathepsin H, the latter being similar to stefin B-papain complex with a few distinct differences. Both, stefin A and stefin B, have been shown to form protein structures known as amyloid fibrils, although stefin A under more harsh conditions than stefin B. Recently, the crystal structure of stefin B tetramer has been determined which involves Pro at the position 74 in a cis isomeric state being essential in stefin B amyloid-fibril formation.

Stefin A was purified from rat skin as a first identified mammalian cystatin and has been found in other epithelial cells, in neutrophils from the liver, in dendritic reticulum cells of lymphoid tissue, in Hassall's corpuscles and in thymic medullary cells. The selective expression of the inhibitor correlates with the tissues participating in the first-line defence against pathogens. Analysis of proteins uniquely involved in the development of the skin and skin immune system revealed strong expression of stefin A in neonatal mouse skin and decreasing with age suggesting an important role in the development of the epidermis.

Stefin B is widely expressed in different cell types and tissues. Subcellularly, it was found mainly in the nucleus of proliferating cells and both in the nucleus and in the cytoplasm of differentiated cells. It has been suggested that stefin B regulates the activity of cathepsin L in the nucleus. Nuclear stefin B interacted with cathepsin L and with histones in the nucleus, but it did not bind to DNA. Increased expression of stefin B in the nucleus delayed cell cycle progression that was associated with the inhibition of cathepsin L in the nucleus. Stefin B could thus play an important role in regulating the proteolytic activity of cathepsin L in the nucleus, protecting substrates such as transcription factors from its proteolytic processing.

Type I cystatins in pathological processes

Stefin A is involved in cellular proliferation and could be a useful target for diseases of abnormal proliferative conditions. Its mRNA level is increased in psoriatic plaques of the psoriasis vulgaris, a common inflammatory disease of the skin, characterized by hyperproliferation of skin cells that ultimately leads to red, scaly plaques. Polymorphism in the gene for stefin A has been associated with atopic dermatitis, a chronic inflammatory skin disease often associated with a defective epidermal barrier. Stefin A is able to protect skin barrier from allergic reactions, including atopic dermatitis. Inhibition of
proteolytic activity of major mite allergens, Der f 1 and Der p 1, by stefin A blocks the up-regulation of IL-8 and GM-CSF release from keratinocytes stimulated with the allergens. 44, 45 Loss-of-function mutations in the gene for stefin A has been identified as the underlying genetic cause of another skin disease, exfoliative ichthyosis. 46

Stefin B was found to form a multi-protein complex specific to the cerebellum with five other proteins and none of them is a protease: the protein kinase C receptor (RACK-1), brain β-spectrin, the neurofilament light chain (NF-L), one protein from the myotubularin family and one unknown protein. Stefin B multiprotein complex is proposed to have a specific cerebellar function and the loss of this function might contribute to the disease in EPM1 patients. 47 EPM1 is a degenerative disease of the central nervous system also known as progressive myoclonus epilepsy of the Unverricht-Lundborg type. Altogether 10 different mutations in stefin B gene underlying EPM1 have been reported, of these the most common change an expansion of a normally polymorphic 12-nucleotide repeat in the promoter region is found that is associated with reduced protein levels. 48 Five different mutations in the coding region of the stefin B gene were found causing protein truncation (R68X), frameshift (K73fsX2) and missense mutations (G4R, Q71P and G50E). 49-53 Stefin B normally localizes in the nucleus, cytoplasm and also associates with lysosomes. The K73fsX2-truncated mutant protein localizes to cytoplasm and nucleus, whereas R68X mutant is rapidly degraded. Two missense mutations, G4R affecting the highly conserved glycine, critical for cathepsin binding, and Q71P, fail to associate with lysosomes. These data imply an important lysosome-associated function for stefin B and suggest that loss of this association contributes to the molecular pathogenesis of EPM1. 53

Accumulation of protein aggregates characterize many neurodegenerative diseases, including Alzheimer’s disease (AD), Parkinson’s disease, dementia, multiple system atrophy, Huntington’s disease, and the transmissible "prion" dementias. 54, 55 Amiloid-β (Aβ) is a soluble peptide, but can form aggregates, either oligomeric or fibrillar that are neurotoxic in AD. 56 Stefin B has been found to be an Aβ-binding protein thus it is likely to have a role in AD. It interacts with Aβ in vitro and in cells and is supposed to have a "chaperone-like" function with binding the Aβ and inhibiting its fibril formation. 57

Type II Cystatins

Among type II cystatins, the most prominent cystatins in immune cells are cystatins C and F, the former being the most abundant human cystatin. Cystatin C was discovered first as a ‘post-γ-globulin’ or ‘γ-trace’ and was the first cystatin determined for amino acid sequence. 58 Later it was shown that its amino acid sequence was highly similar to cystatin, isolated from chicken egg white. 59 Mature human cystatin C is composed of 120 amino acid residues and has a molecular mass of 13,343 Da. The cystatin C cDNA sequence revealed that cystatin C is synthesized as a preprotein with a 26 residue signal peptide.60 The gene, encoding cystatin C is typical house-keeping gene type, which is expressed in a variety of human tissues and cells. However, like the most of other type II cystatins, cystatin C is secreted and can be found in high concentrations in body fluids, in particular high levels have been found in seminal plasma and cerebrospinal fluid. Cystatin C is a strong inhibitor of all papain-like proteases (clan C1) 61, 62 and asparaginyl endopeptidase/legumain (clan C13) 63 and could be seen as a major human extracellular cysteine protease inhibitor. Cystatin C has been suggested as regulating cathepsin S activity and invariant chain (II) processing in dendritic cells (DCs), however, further studies excluded a role in controlling MHC II-dependent antigen presentation in DCs. 65 Additionally, the maturation process of DCs leads to reduced levels of cystatin C and colocalization studies do not support intracellular interactions among cystatin C and its potential target enzymes cathepsins H, L and S in immature or mature DCs. 66 A better candidate for regulating the proteolytic activity of cysteine proteases within the DCs was shown to be cystatin F. 67

Cystatin F was discovered by three independent groups. Two of them identified the new inhibitor by cDNA cloning and named it leukocystatin and cystatin F 68,69. The third group found overexpressed mRNA encoding cystatin F in liver metastatic tumors and identified it as CMAP (cystatin-like metastasis associated protein). 70 Human cystatin F is synthesized as a 145 amino acids pre-protein with a putative 19 residues signal peptide. 68, 69 Although it is made with a signal sequence, only a small proportion is secreted 69 and importantly, it is secreted as a disulfide-linked dimer 71 which is inactive until it is reduced to its monomeric form. 72 It is glycosylated 68,69 and mannose-6-phosphate modification of its N-linked saccharides is used for targeting to the endosomes and lysosomes. 73 Glycosylation at Asn62 is proposed to protect the intermolecular disulfide from reduction, explaining unusually strong reducing conditions needed to monomerize dimeric cystatin F in vitro. 72, 74 Inactive dimer to active monomer con-
version is also achieved with proteolitic cleavage by so far unidentified protease action on the extended N-terminal region of cystatin F.\(^7^5\) Being glycosylated, inactive cystatin F can be internalized through the mannose-6-phosphate receptor pathway and activated within different cells.\(^7^3\) All these facts strongly imply on intracellular action of cystatin F as well as on “in trans” activity of its secreted inactive inhibitor which can be internalized and activated inside another cells. Cystatin F tightly inhibits cathepsins F, K, V, whereas cathepsins S and H are inhibited with lower affinities and cathepsin X is not inhibited at all.\(^6^9,\)\(^7^6\) C13 cysteine protease involved in antigen processing, mammalian legumain or asparaginyl endopeptidase (AEP), is also inhibited by cystatin F although it shows reduced affinity for AEP compared with cystatins C and E/M.\(^6^9\) The inhibitor is expressed selectively in immune cells such as cytotoxic T cells, natural killer cells (NK cells), monocytes, DCs (Figure 1).\(^6^7,^6^9,^7^7,^7^8\) Its levels and localization are controlled according to the physiological state of the cells. It is strongly up-regulated in monocyte-derived DCs undergoing LPS-induced maturation and downregulated in TPA (causing monocytic differentiation towards a granulocytic pathway) or ATRA (causing monocytic differentiation towards macrophages) stimulated U937 cells.\(^7^7,^7^9\) The unique features of cystatin F suggests that this immune-cell specific inhibitor plays a role in immune response-related processes through inhibition of specific enzyme targets, even though the details of its role remain unexplained. It is likely that in DCs, cystatin F could regulate the activity of cathepsin L and thus controlling the processing of procathepsin X, which promotes cell adhesion via activation of Mac-1 (CD11b/CD18) integrin receptor.\(^6^7\) One of the protease targets of cystatin F is cathepsin C\(^7^5\), the cysteine protease that activates the granzymes in cytotoxic T cells, NK cells and several effector proteases of neutrophils and mast cells.\(^7^5,^8^0,^8^1\) Cystatin F is a strong inhibitor of cathepsin C only as an N-terminally truncated form.\(^7^5\) Our recent work suggests potential regulation of split anergy in NK cells through inhibition of cathepsin C and consequently downstream regulation of granzymes (Manuscript in prep). Recent work by Dr. Jewett and colleagues indicate that induction of split anergy in NK cells may be an important physiological step required for the conditioning of the NK cells to support differentiation of the stem cells. In this regard they proposed that conditioned or energized NK cells may play a significant role in differentiation of the cells by providing critical signals via secreted cytokines as well as direct cell-cell contact. To be conditioned to drive differentiation, NK cells may have to first receive signals through their key surface receptors either from stem cells or other immune effectors or fibroblasts in the inflammatory microenvironment and lose cytotoxicity and gain cytokine producing phenotype (split anergy). These alterations in NK cell effector function will ultimately aid in driving differentiation of a population of surviving healthy as well as transformed stem cells. Regulation of cystatin F and consequently cathepsin C and granzymes by NK cell surface receptors could be the mechanism which conditions NK cells to undergo split anergy and become regulatory NK cells.

**Figure 1: Lysosomal localization of cystatin F in adherent dendritic cells (DCs).** Part of image is magnified in lower right corner. The white colour indicates colocalization of two labelled antigens, confirming the presence of cystatin F in lysosomes. The threshold value for colocalization was set to one half of the maximal brightness level. The mask of the pixels above the threshold in both channels (significant colocalization, blue colour) and the contour plot are shown.\(^6^7\)
For cystatin M a downregulated mRNA in metastatic breast tumor cells was identified by differential display when compared to normal and primary breast tumor cells. Independently, the same molecule was found by others in cDNA libraries derived from epithelial cells, and was designated cystatin E. Cystatin E/M is a 14.5 kDa secreted protein that has an overall structure similar to other family II cystatins, such as a signal peptide and two intrachain disulfide bonds. Like cystatin F, it possesses the unusual characteristic of being a glycoprotein, carrying an N-linked carbohydrate chain at position 108. This protein is only distantly related to the other known family members as reflected by the genomic position of the cystatin E/M gene on chromosome 11q13, whereas all other family II cystatin genes are clustered in a narrow region on chromosome 20p11.2. Cystatin E/M is synthesized as a pre-protein with a putative 28 residues while mature cystatin E/M contains 121 amino acids. High expression levels are largely confined to skin epithelia, which emphasizes its prominent role in cutaneous biology. Its expression was also recently reported in breast tissue and in oligodendrocyte-astrocyte-like cells of human brain. Cystatin E/M is a high-affinity inhibitor of cathepsins V, L and asparaginyl endopeptidase/legumain, with lower affinity it binds also cathepsin B. In vivo mouse models have revealed that cystatin E/M is a key molecule in a biochemical pathway that controls skin barrier formation. The terminal stage of the differentiation process of keratinocytes is desquamation, which involves degradation of lipids in the intercellular spaces and loss of residual intercellular desmosomal connections. Cells reaching the skin surface are continuously sloughed and replaced by inner cells differentiating and moving outward. Cystatin E/M could have a role in desquamation by the regulation of cathepsin V protease activity or could regulate cathepsin L and legumain activities cosslinking of structural proteins by transglutaminase 3 in the keratinocyte differentiation process of the epidermis and the hair follicle. In addition, it might also be involved in the regulation of cathepsin D activity, as it is known that cathepsin L can process and activate cathepsin D and mature cathepsin D is also important in the desquamation process of the skin.

The studies regarding implication of other type II cystatins in immune system are not numerous. Cystatins S, SN and SA were first isolated from human saliva and later identified as a cysteine protease inhibitors and their role in controlling cysteine proteases derived from bacteria has been suggested. These three proteins with similar sequence (88% identity at the protein level) are distantly related to cystatin D (less than 60% identity at the protein level with cystatins S, SN and SA). Although they are possessing inhibitory activity, they are all poorer inhibitors of cysteine cathepsins than cystatin C. Inhibition of bacterial cathepsins have been tested by cystatins C, SN, S and chicken cystatin and they are generally inactive against bacterial cysteine proteases. In contrast to bacterial cysteine proteases, they are more efficient against cysteine proteases from parasits; for example, cystatins SA and SN are inhibitors of trypanosome cruzain and chicken cystatin inhibits congopain from Trypanosoma congolense and cruzipain, the major cysteine protease of the protozoan parasite Trypanosoma cruzi. These results suggest a possible defensive role for the host's cystatins after parasite infection. Cystatin SN has been found also in dendritic cells exposed to Toxoplasma gondii and it could thus modulate antigen presentation. Salivary cystatins may have a role in the host defense mechanism against virus infections as they can interfere with events in viral replication. Cystatin D has been found to be a potent inhibitor of coronavirus replication and cystatins S, SA and SN can suppress the infectivity of adenovirus and herpes simplex virus 1.

Human genome contains several related genes encoding gycoproteins that possess sequence similarity with other type II cystatins. The shared characteristic of three subgroup members is an expression pattern limited primarily to the male reproductive tract. Testatin and cystatin T are specifically expressed in the testis and cystatin-related epididymal spermatogenic protein (CRES) exhibits highly tissue-specific expression in the reproductive tract. Although they possess a typical C-terminal PW motif, they lack the N-terminal glycine and motif QXVXG important for inhibition of cysteine proteases.

**Type II cystatins in pathological processes**

Changes in cystatin C expression and localization have been associated with various neurodegenerative pathologies. For example, a point mutation in the cystatin C gene, resulting in the substitution of Leu to Gln, is responsible for the dominantly inherited icelandic type of amyloidosis, hereditary cystatin C amyloid angiopathy (HCCAA). Patients with this disease suffer from successive brain hemorrhages concluding in death at the age of 30. In first line the formation of cystatin C aggregates are responsible for the outcome and progression of the disease, however,
the loss of inhibitory function due to aggregation and lower concentration in brain can not be excluded. 116 The levels of cystatin C were highly reduced in cerebrospinal fluid of individuals suffering from multiple sclerosis (MS) compared with healthy individuals. Its low concentration suggests that the inhibition of cysteine proteinases is impaired in this disease; hence higher activity of cysteine proteinases could initiate or increase the breakdown of myelin. 119 Cystatin C was shown to be implicated in Alzheimer’s disease (AD). It co-deposits with amyloid-beta (Aβ) in amyloid plaques of AD patients 120, associates with Aβ and inhibits Aβ oligomerization in vitro and in vivo. 121, 122 Thus, cystatin C could protect the brain from amyloid-induced toxicity and may have therapeutic implications for AD. 56 However, there are opposing results demonstrating neuronal cell death upon injecting cystatin C into the brain of AD mouse model, 123 showing that cystatin C triggers Aβ accumulation by inhibiting cathepsin B-induced Aβ degradation. 124 Cystatin C may have an important role in brain injuries as its enhanced expression has been observed in response to different types of insults to the brain, such as ischemia and epilepsy. 125-127

Regarding constant serum concentrations of cystatin C it is a suitable marker for glomerular filtration rate (GFR) and kidney function and thus has a possible application as a replacement for creatinine. 128, 129 Studies on the sensitivity and specificity of cystatin C for detecting impaired GFR strongly suggest it to be superior diagnostic marker to creatinine for detecting impaired GFR. 130 However, caution is necessary in cases of patients with malignancy or other diseases with increased activity of cysteine proteinases which exhibit increased cystatin C levels irrelevant to kidney function. 131

It has been suggested that disturbance of the cystatin E/M-cathepsin pathway could contribute to dysregulated skin barrier function as observed in the inflammatory dermatoses. Atopic dermatitis and psoriasis are two common chronic inflammatory skin diseases in which the expression of many genes and the formation of the epidermal barrier are altered. Recent genetic studies have revealed that abnormalities in epithelium-expressed genes are an important etiological factor. 132-134 The study Cheng et al. 86 have shown decreased mRNA and protein expression levels of cystatin E/M and cathepsin V in the inflamed skin during atopic dermatitis and psoriasis, which suggests their prominent role in these inflammatory diseases.

Expression of cystatin F has been associated to some pathological conditions. It was found to be massively expressed in the microglia, mono-
associated proteolytic activity. Outside the cells, higher levels of type II cystatins may impair extracellular activity of cysteine proteases, associated directly or indirectly with the degradation of ECM and resulting in tumor cell invasion and metastasis. However, higher levels of cystatins in body fluids have been associated with poor prognosis in cancer patients supporting their role in regulation of proteases involved in the regression of tumors. In melanoma and colorectal cancer, increased extracellular levels of cystatin C as well as stefins correlated significantly with high risk of adverse outcome in cancer patients (Figure 2). Cathepsin B/cystatin C complex was also found to be less abundant in sera of patients with tumors suggesting an imbalance between the enzyme and its inhibitor in cancer patients. 141

Animals with excluded expression of type I or type II cystatins experience better outcome with regard to tumor growth and metastasis as the wild type ones. These contradictory results can be explained by the fact that type II cystatins are involved also in processes resulting in tumor regression such as anti-tumor immune response, apoptosis, cell migration and seeding. 9 In particular, the role of cysteine proteases is very important for proper maturation of antigen presenting cells, antigen processing and the presentation to T cells, therefore, the enhanced inhibition may affect the activation of naive T cells by tumor associated antigens and impair T cell dependent anti-tumor immune response, known to be effective for eradication of tumor cells. Higher levels of cystatins may affect also the innate immunity, as mentioned above, increased levels of cystatin F inactivate cathepsin C and thus impair activation of granzymes and cytotoxicity of NK cells. 75,142

On the other hand, patients with higher local levels of stefins A and B in non-small cell lung tissue than in control lung tissue exhibited a better survival probability implying on their ability to counteract harmful tumor-associated proteolytic activity. 143 Also, the overexpression of cystatin C in tumor cells inhibited melanoma metastasis formation. 144 Thus, besides the concentration, cell and tissue localization of cystatins could make a critical switch between harmless and harmful and their application as anti-cancer agents has to be considered with caution and should be directed specifically to cysteine proteases which promote specific stage of tumor progression. For instance, the expression and the role of cystatin E/M in tumorigenesis may differs depending on the stage of the cancer. Since it was initially identified as a downregulated mRNA in metastatic breast tumor cells compared to normal and primary breast tumor cells 85, it is likely that the loss of the expression of cystatin E/M is associated with the progression of a primary tumor to a metastatic phenotype. Furthermore, scid mice orthotopically implanted with cystatin E/M-expressing MDA-MB-435S breast cancer cells show significantly delayed primary tumor growth compared to controls, the incidence of metastasis, however, appeared to be unaltered, confirming that cystatin E/M suppressed tumor cell proliferation at the secondary site. 145 Higher level of cystatin F mRNA in the colorectal cancer tissue correlated both with liver metastasis and with worse patient prognosis. 146 Therefore, it is possible that a determination of the cystatin F mRNA expression may help in the identification of patients at high risk for metastasis, and these patients could thereby benefit from careful examinations and extensive treatments.

The pathways by which cystatins modulate immune response unrelated to their function of cysteine protease inhibitors, cannot be excluded, as shown for cystatins of filarial nematodes 147, chicken cystatin 148,149 and cystatin C. 150 The latter has been shown to antagonize TGF-β binding to the cell surface receptors of normal and cancer cells by interacting physically with the TGF-β type II receptor and abrogating the binding of TGF-β. TGF-β is a multifunctional cytokine endowed with both tumor-suppressing and tumor-promoting activities. In normal cells TGF-β inhibits proliferation and induce apoptosis, however during the transformation to cancer cell it becomes a trophic factor for the transformed cells promoting their proliferation. 151,152 Interestingly, down regulation of tumorigenesis through a similar mechanism has been shown also for a family-3 cystatin (AHSG) which inhibits colon carcinogenesis by suppressing TGF-β signal transduction by blocking TGF-β1 binding to cell surface receptors. 153 There is an evidence indicating that cystatin E/M is a tumor suppressor factor important in breast and brain malignancy reducing tumor cell proliferation, its expression is markedly decreased in carcinoma cells. 87,88,145,154

In the study Shridhar et al. 155, the effects of cystatin E/M expression on malignant properties were examined on human breast carcinoma MDA-MB-435S cells, which are highly tumorigenic and metastatic and normally do not express cystatin E/M. The constitutive cystatin E/M expression in transfected cells resulted in a significant reduction of cell proliferation, migration, Matrigel invasion, and adhesion to endothelial cells. Cell migration and matrix invasion appeared to be controlled by the lysosomal cysteine proteases, as both recombinant cystatin E/M and E-64 were able to block these processes. In contrast, reduction of cell proliferation
and adhesion to an endothelial cell monolayer were both independent of the inhibition of lysosomal cysteine proteases. Cystatin E/M could thus act through various mechanisms, besides inhibition of cysteine proteases also by inhibition of legumain and modulation of gene transcription. The latter is confirmed by the fact, that cystatin E/M expression in MDA-MB-435S cells significantly changed the expression of extracellular matrix components, cytokines, kinases, and phosphatases, as well as several key transcription factors, including downregulation of autotaxin, a signaling molecule that has been linked to breast cancer invasiveness. Furthermore, epigenetic studies reported DNA methylation-dependent silencing of the cystatin E/M gene in breast cancer cell lines and in primary breast tumors. Hypermethylation was significantly associated with gene silencing and loss of cystatin E/M protein expression, whereas enhanced gene expression was measured in breast cancer cells cultured on the DNA demethylating agent. Collectively these data suggest that cystatin E/M could act as a tumor suppressor through various mechanisms.

Conclusions

In conclusion, cystatins are involved in a number of normal and pathological conditions. They exert several immunomodulatory functions by controlling the activity of cysteine proteases or by other mechanisms not related to their inhibitory function. They may contribute to the proteolytic processing of progranzymes and other substrates, major histocompatibility complex class II antigen presentation, maturation of dendritic cells, modulation of integrin function and formation of the skin barrier. It has become evident that disturbances in expression and localization of cystatins may be implicated in several pathological processes, such as inflammatory skin diseases and neurodegenerative disorders. In particular for stefin B and cystatin C, association with amyloid-beta in Alzheimer’s disease has been demonstrated. In cancer, inhibitory effect of cystatins could beneficially counteract tumor-associated proteolytic activity or, could also be harmful when impairing the functions in cysteine cathepsins in anti-tumor immune response and is strongly depended on their cell and tissue localization.

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Competing Interests

The authors have declared that no competing interest exists.
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