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REVIEW

Cystatin C is a disease-associated protein subject to multiple regulation

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A protease inhibitor, cystatin C (Cst C), is a secreted cysteine protease inhibitor abundantly expressed in body fluids. Clinically, it is mostly used to measure glomerular filtration rate as a marker for kidney function due to its relatively small molecular weight and easy detection. However, recent findings suggest that Cst C is regulated at both transcriptional and post-translational levels, and Cst C production from haematopoietic cell lineages contributes significantly to the systematic pools of Cst C. Furthermore, Cst C is directly linked to many pathologic processes through various mechanisms. Thus fluctuation of Cst C levels might have serious clinical implications rather than a mere reflection of kidney functions. Here, we summarize the pathophysiological roles of Cst C dependent and independent on its inhibition of proteases, outline its change of expression by various stimuli, and elucidate the regulatory mechanisms to control this disease-related protease inhibitor. Finally, we discuss the clinical implications of these findings for translational gains.
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Cystatin C (Cst C) is a potent extracellular inhibitor of cysteine protease, and has been generally considered a ubiquitously expressed protein as no obvious regulatory elements were found in its gene promoter.[1] Clinically, Cst C is mostly used as a biomarker of kidney function for its relatively lower molecular weight (~13.3 kDa) and easy detection compared to the injection of compounds, radioisotopes or radiocontrast agent[2] to measure glomerular filtration rate (GFR), an index of kidney health, because Cst C is removed from the blood stream by glomerular filtration, whose decline as a result of failed kidney function will lead to increased serum Cst C concentration. Cst C serum levels were claimed to be a more precise index of kidney function than that of creatinine[3] under the assumption that serum input of Cst C is constant, and the main determinant of blood Cst C levels is the rate at which it is filtered at the glomerulus. Furthermore, recent advances have facilitated the use of Cst C as a clinical measure of kidney function (the update on Cst C in recent kidney disease guidelines has been reviewed elsewhere[4]). Although, the bulk of literature report the use of Cst C for GFR estimation, precaution should be taken when the outcomes of this measurement is interpreted because recent studies indicated that both genetic polymorphisms[5,6] and clinical interference[7,8] could make Cst C an unreliable index of GFR. Indeed, accumulating reports have documented that subjected to the influence of many factors, serum Cst C levels do vary independent of renal functions, and the synthesis and secretion of Cst C seem to be tightly regulated under different pathophysiological conditions. For example, body composition, thyroid function, glucocorticoid and C-reactive protein levels or even cigarette smoking and pregnancy status of the candidates examined have been found to affect Cst C blood concentration,[9,10] let alone its alteration in patients with cancer, HIV infection, cardiovascular diseases and neurological disorders.[11–13] Moreover, new studies reported direct involvement of Cst C in many pathogenic processes other than renal disorders.[14,15] Thus, the oscillation of blood Cst C levels could actually reflect the change of Cst C production, consumption, inactivation or fibrillation (mentioned later) rather than its filtration in the kidney, and the readout of plasma Cst C concentration might have different clinical implications. In this review, we first summarize the major pathophysiological roles of Cst C to illustrate its importance as a functional protein in the body rather than an ubiquitously expressed measuring substance. Second, we outline the impact of different stimuli on its expression and elucidate some of the latest developments on the regulatory mechanisms to explain Cst C variation under certain circumstances. In the end, the clinical implications derived from these studies are also discussed.

PATHOPHYSIOLOGICAL ROLES OF CST C

Functions dependent of its inhibition of proteases

Cysteine cathepsins play fundamental roles in multiple biological processes such as protein turnover, pro-protein processing, bone remodeling, antigen presentation and apoptosis.[16] They are also involved in numerous pathological processes such as cardiovascular disease and inflammation.[13] The activities of these enzymes both inside and outside of cells thus need to be tightly controlled by their endogenous inhibitors, of which Cst C is the most abundant and potent member.

Intracellular roles

Apoptosis. Intracellular lysosomes undergo membrane permeabilization in response to extra- or intra-lysosomal stimuli, and the

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involvement of lysosomal cysteine proteases in apoptosis has been confirmed in several systems.[17,18] However, the roles of the endogenous lysosomal cysteine protease inhibitor Cst C in apoptosis remain controversial. Upregulation of Cst C expression was found to correlate with oxidative stress-induced apoptosis in cultured rat neurons,[19] and Cst C injection into rat hippocampus led to neuronal cell death in the granule cell layer of dentate gyrus *in vivo*,[20] indicating a possible functional role of Cst C in apoptosis induction. Since, the Cst C-induced neuronal cell death could be inhibited by simultaneous co-application of cathepsin B, the inhibitory activity of Cst C on a cysteine protease was proposed to be involved in the process of apoptotic neuronal cell death.[20] Recent studies indicated that the roles of Cst C in neuronal cell apoptosis induction include decreasing B-cell leukemia-2 (Bcl-2) and increasing active caspase-9 protein levels via the Jun N-terminal kinase (JNK)-dependent pathway,[21] and upregulation and accumulation of the insoluble α-synuclein in oligodendrocytes and neurons.[22] Interestingly, apart from these documented pro-apoptotic roles, Cst C was also found to have anti-apoptotic effects on neuronal cells. For example, expression of Cst C in PC12 cells derived from a pheochromocytoma of rat adrenal medulla prevents oxidative stress-induced death *in vitro*.[23] In accordance with this finding, the anti-apoptotic function of Cst C was further demonstrated *in vivo* in a mouse model of the inherited neurodegenerative disorder, progressive myoclonic epilepsy type 1, where loss of function in cystatin B and enhanced cathepsin B and D activities are the underlying pathologies. Crossbreeding of the mice with either Cst C-overexpressing transgenic or Cst C-deficient mice revealed that Cst C levels *in vivo* can affect neuron degeneration caused by the proteases, indicating that Cst C partially prevents neural cell death *in vivo* through inhibition of cathepsins activity.[24] (Described in the section of Neuroprotective roles.)

Antigen presentation. Proteolysis of antigen by cysteine cathepsins to generate immunogenic peptides and guide the transit of both major histocompatibility complex class II (MHCII) and MHC-like molecules through the endocytic compartments are important intracellular activities in antigen-presenting cells. As a potent inhibitor of cysteine cathepsins, Cst C had initially been implicated in playing a regulatory role in the developmental control of MHCII presentation in dendritic cells (DCs) by inhibiting cathepsin S in invariant chain cleavage.[25] Consistent with the role of Cst C in antigen presentation, a cystatin homolog produced by the filarial nematode parasite was found to inhibit lysosomal cysteine protease activities, which subsequently impeded the generation of human B cells.[26] Further experiments using bone marrow-derived DCs indicate that interleukin 6 (IL-6)-mediated signal transducer and activator of transcription 3 (STAT3) activation decreased Cst C expression and MHCII αβ dimer levels.[27] However, a separate study using mouse primary DCs isolated from Cst C-deficient mice demonstrated that Cst C is neither necessary nor sufficient to control MHCII expression and antigen presentation in DCs.[28] These discrepant results obtained from different laboratories could be due to the different cell types investigated, and/or to the compensatory roles played by other cysteine protease inhibitors in the absence of Cst C. In support of this view, cell-specific regulation of cathepsin activity by Cst C was identified in two similar antigen-presenting cells in the brain: Cst C was found to inhibit cathepsin L activity in astrocytes, but does not regulate cathepsins L and S in microglia.[29]

When intracellular localization of the Cst C and its target proteases were examined in human DC differentiated from monocytes *in vitro*, the different compartmentation of Cst C and cathepsins S, L and H in immature and mature DCs suggests that the regulatory potential of Cst C toward these cathepsins inside DCs is limited, which could explain the inconsistent findings related to the intracellular roles of Cst C. Instead, large secretion of Cst C over cathepsins S, L and H was observed in the culture media,[30] indicating that the extracellular compartment is the primary site for these interactions.

Extracellular roles. The structure of Cst C predisposes this protease inhibitor to extracellular functions as a secreted protein. Accordingly, Cst C is found in all body fluids at significant concentrations,[31] which makes it a major regulator of cysteine protease activity in the extracellular medium.

Atherosclerosis. Atherosclerosis-based vascular disease is an inflammatory disease characterized by extensive remodeling of the extracellular matrix of the arterial walls. Apart from the well-known matrix metalloproteinases and serine proteases, lysosomal cysteine proteases were also found to be involved.[32] Present in substantial amounts in the normal vessel walls, the expression of Cst C was found to be severely reduced in both atherosclerostic and aneurysmal lesions, and increased abdominal aortic diameter correlated with lower serum Cst C levels in humans.[13] Furthermore, the pathogenic roles of Cst C were tested in a model of atherosclerosis-prone mice with apolipoprotein E deficiency ($apoE^{-/-}$) where the elevated cathepsins were associated with the atherosclerostic process.[33] In two independent studies, Cst C- and apoE-double-deficient mice were generated, both of them confirmed an anti-atherosclerostic function of Cst C in the $apoE^{-/-}$ mice, although differences regarding lesion size and composition were found.[34,35] These differences are most probably caused by differences in the duration of high-fat diet of the mice, sex of the mice and anatomic site of analyzed lesions. Interestingly, in line with the anti-atherosclerostic role of Cst C in this animal model, polymorphisms in the promoter regions of the Cst C gene were found to influence the plasma Cst C concentration in human coronary artery disease,[36] indicating the production of this protease inhibitor could be subject to regulation under the diseased conditions.

Tumor metastasis. Metastatic tumor cells invade host tissues through a series of steps that require proteolytic enzymes including cysteine cathepsins to degrade components of the extracellular matrix.[37] As a most abundant endogenous cysteine proteinase inhibitor, Cst C is believed to prevent tumor progression by inhibiting the activities of a family of lysosomal cysteine cathepsins. Results from the first Cst C-deficient animals indicated that Cst C concentration *in vivo* might influence tumor metastasis in some tissues.[38] In agreement, the reduced Cst C levels correlate with increased metastasis of different tumors in human tissues or patients.[39] Furthermore, local over-expression of Cst C in the host tissue microenvironment could lead to successful reduction of metastasis via cysteine cathepsin inhibition in an experimental tumor model.[40] This inverse correlation between Cst C and tumor aggressiveness, however, may not always involve inhibition of cysteine proteases. For example, elevated matrix metalloproteinases 2 and crosstalk between Cst C and androgen receptor-mediated pathways were reportedly implicated in prostate cancer invasion and metastasis,[41] indicating multiple roles of Cst C in tumor metastasis.

Pathogen invasion. Although a secreted protein, Cst C was also reported to be up-taken by cells of both foreign and endogenous origins in various tissues to regulate both intracellular and extracellular cysteine protease activities.[42] One of the major roles of cystatins is to protect the host against invading microorganisms and parasites that

use cysteine proteases to enter the body.[43] Chicken cystatin was first reported to partially block poliovirus replication in infected human cells.[44] Further study demonstrated that a small peptide derivative that mimics part of the proteinase-binding center of human Cst C could inhibit a cysteine protease specific for the growth of group A streptococci, blocking the growth of these bacteria both *in vivo* and *in vitro*.[45] Similarly, recombinant human Cst C was also proven to inhibit the growth of herpes simplex virus[46] and human coronaviruses.[47] Moreover, a family of cathepsin L- and B-like cysteine proteases, found in all species of *Leishmania* examined, are required for the parasite growth and virulence.[48] Since, these parasite cysteine proteases may not only digest the host extracellular matrix to facilitate their invasion, but also help to ensure a Th_2-like response led to parasite proliferation,[49] cystatin treatment in combination with interferon γ (IFNγ) that leads to reduced parasite numbers, successful Th_2 to Th_1 conversion, and NO generation, which finally resulted in abrogation of parasite infection in a mouse model of leishmaniasis.[50]

Functions independent of its inhibition of proteases

Modulating roles. Most abundantly expressed in tissues, Cst C also serves numerous functions independent of protease inhibition including affecting signaling properties of other molecules. For example, Cst C antagonizes the binding of transforming growth factor-beta (TGF-β) to its cell receptors by physically interacting with TGF-β type II receptor independent of its protease inhibitory activity, as overexpression of Cst C mutant that is impaired in its ability to inhibit cathepsin activity blocked TGF-β-dependent invasion of 3T3-L1 fibroblasts.[51] This novel function of signaling modulation allows Cst C to inhibit the oncogenic activities of TGF-β through stimulation of mammary epithelial-mesenchymal transition.[52] Apart from blocking interaction between TGF-β and its receptor, Cst C was also found to prevent TGF-β signaling partly by reducing the extent of mothers against decapentaplegic homolog 2 (Smad2), p38 mitogen-activated protein kinases (p38 MAPKs) and extracellular signal-regulated kinases 1/2 (Erk1/2) phosphorylation in murine 4T1 breast cancer cells.[53] In addition to affecting TGF-β signaling, Cst C is involved in the IFNγ signal transduction pathway. It activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65, induces NO synthase, but downregulates IL-10 in macrophages.[54]

Through interacting with other proteins with effects on their signaling properties, Cst C can also affect biological functions of cells. During brain development, Cst C was identified as a factor to upregulate the glial fibrilllary acidic protein promoter.[55] As a result, addition of human Cst C to the culture medium of primary brain cells increased the number of glial fibrilllary acidic protein-positive cells and neurospheres formed from the embryonic brain. Again, the promotion of astrocyte development by Cst C appears to be unrelated to its protease inhibitor activity, as another cysteine protease inhibitor did not have this effect.[56] Along the same line, a recent study identified a novel function of Cst C in mediating amyloid β (Aβ) precursor protein-induced proliferation of neural stem/progenitor cells.[57] Although the mechanism of Aβ precursor protein in stimulating neural stem/progenitor cells to secrete Cst C needs further characterization, this study contributes to a better understanding of multifunctional roles of Cst C in the pathogenesis of Alzheimer's disease.

Amyloidogenic roles. In addition to cysteine protease inhibition, Cst C is also one of the few amyloidogenic proteins that form a fibrillary structure deposited in the vascular walls, affecting the health of blood vessels (angiopathy). The proteins identified in cerebral amyloid angiopathy include beta/A4, transthyretin and Cst C.[58] Cst C amyloidogenesis begin with dimerization by a process known as 'three-dimentional domain swapping', in which two parts of the cystatin structure become separated from each other and next exchanged between two molecules.[59] Interestingly, with their inhibitory region hidden within the dimer interface, Cst C dimers cannot inhibit cysteine proteases.[60] This none-inhibitory Cst C dimer is required for the formation of the Cst C oligomers, intermediates in fibrillogenesis, because variants of monomeric Cst C, stabilized against domain swapping to block the inhibitory site, fail to produce oligomers, indicating that Cst C fibrils are formed by propagated domain swapping.[61]

Domain swapping must be preceded by at least partial unfolding of the molecule,[62] therefore, any factors affecting the stabilization of the molecule could initiate the domain swapping process. For instance, the point mutation with substitution of native leucine in position 68 by glutamine (L68Q) disrupts a network of the hydrophobic interactions, which leads to increased tendency for dimerization and aggregation *in vitro*.[63] The L68Q replacement is also a naturally occurring point mutation in the Cst C protein sequence with autosomal dominant hereditary. This Cst C variants form fibrils *in vivo* in the brain vasculature, which cause hemorrhage, dementia and eventually death in people carrying this mutation, a condition known as hereditary cystatin C amyloid angiopathy,[64] also called hereditary cerebral hemorrhage with amyloidosis, icelandic type.[65] The cellular transport of Cst C is impeded by the mutated L68Q variants, resulting in diminished Cst C levels in cerebrospinal fluid[66,67] and retained Cst C mutants in blood monocytes from patients.[67] Consistently, *in vitro* studies demonstrated that clones expressing the gene encoding L68Q Cst C secreted either lower amounts of Cst C,[68] or unstable protein susceptible to a serine protease,[69] contributing to the reduced extracellular Cst C levels.

Cst C amyloid fibrils not only affect brain vasculature but also may lead to toxicity in other tissues. As the most abundant protein, Cst C was found in tissues outside of the brain including the testis, and so was the L68Q variant.[70] A recent study reports that heterozygous transgenic mice that express this pathogenic variant were unable to generate offspring, indicating the L68Q Cst C amyloid affects sperm function.[71] Further analysis of the L68Q mice demonstrated that their epididymal spermatozoa were unable to fertilize oocytes and exhibited poor sperm motility in the presence of Cst C amyloid that were not found in the wild-type mice. The L68Q epididymal fluid, when depleted of the Cst C amyloids, however, did not impair the motility of wild-type spermatozoa, suggesting that amyloids in the epididymal fluid can be cytotoxic to the maturing spermatozoa resulting in male infertility.[71] However, two other groups in earlier studies have also generated the transgenic lines expressing this variant form of Cst C, but they were live and fertile,[72,73] questioning whether the pathology described is really brought about by the Cst C variants or the site where the transgene was inserted.

Neuroprotective roles. Although mutant Cst C is toxic by amyloidosis, wild-type Cst C has multiple neuroprotective roles (comprehensively reviewed by Gauthier *et al.*[74]). An amyloidogenic protein itself, Cst C has an anti-toxic role against another amyloid protein, Aβ. Studies *in vitro* demonstrated that Cst C binds to Aβ and inhibits its oligomerization[75] and amyloid fibril formation.[76] Furthermore, in Aβ precursor protein transgenic mice, Cst C was found to physically associate with the soluble, non-pathological form of Aβ *in vivo*, which inhibited the aggregation and deposition of Aβ plaques in the brain.[73,77] Moreover, Cst C can also directly protect neuronal cells from amyloid toxicity, as extracellular addition of human Cst C

promoted the survival of cultured neuronal cells against the preformed oligomeric or fibrillar Aβ.[78]

Autophagy is important for the survival and homeostasis of neurons as they cannot dilute accumulating detrimental substances or damaged organelles by cell division (see latest review[79] for details). Cst C was first reported to induce a fully functional autophagy to protect neuronal cells against various stress via the mammalian target of rapamycin (mTOR) pathway *in vitro* independent of its inhibitor activity.[80] Consistently, following experimental subarachnoid hemorrhage, exogenous Cst C administration was recently found to activate autophagy pathway *ex vivo*, which plays a beneficial role in early brain injury in a rat model.[81,82] In addition to these apoptotic factors like nutritional deprivation, oxidative stress and hemorrhage, another neurotoxic element that can be counterbalanced by Cst C partly through autophagy is mutant Cu/Zn superoxide dismutase, a frequent cause of inherited amyotrophic lateral sclerosis.[83]

REGULATION OF CST C BY DIFFERENT STIMULI

The numerous pathophysiological roles of Cst C foretell that the original application of this small molecular weight (MW) protein as a measurement of the GFR for kidney function might no longer be appropriate, as participation in various pathophysiological processes could lead to consumption and subsequent regulation of this multifunctional protease inhibitor. Indeed, Cst C levels could be altered by many common stimuli under both physiological and diseased conditions.

Inflammatory cytokines and pathogens

Inflammation is a quite common condition caused by various pathogens that elicit the burst of inflammatory cytokines by the host as a first line of defense. The influence of inflammatory cytokines on the production of Cst C was documented almost 30 years ago. Treatment of resident mouse peritoneal macrophages *in vitro* with the bacterial compound lipopolysaccharide (LPS) or pro-inflammatory IFN-γ downregulates Cst C secretion.[84] Likewise, inflammatory cytokine IL-6 signaling *in vivo* was found to decrease Cst C expression in DCs.[27] Moreover, human immunodeficiency virus infection could either inhibit Cst C expression in DCs[12] or reduce the reactivity of Cst C with its target enzyme cathepsin B in macrophages.[85] Along the same line, we found that in an inflammatory mouse model created by intravenous injection of CpG oligodeoxynucleotides, mimics of bacterial and viral DNA responsible for immune stimulation, the synthesis of Cst C in DCs as well as the circulating pools of Cst C in blood were greatly reduced.[86] In non-hematopoietic cells, however, addition of periodontal pathogens and pro-inflammatory cytokines to human gingival fibroblasts was found to enhance their Cst C expression,[87] and upregulated levels of Cst C was observed in the ethmoid sinus mucosa of patients with chronic sinusitis,[88] indicating different roles and/or regulatory mechanisms of Cst C might exist in different cell types and tissues. However, the substantial impact of pathogens on immune cells, and our recent finding that serum levels of Cst C could be significantly affected by the replacement of bone marrows[89] highlights the important impact of altered Cst C production from hematopoietic cells on the systematic pools of Cst C during inflammation.

Growth factors and hormones

Growth factors or hormones are naturally occurring substance capable of regulating a variety of cellular processes by stimulating cellular growth, proliferation, healing and differentiation. They are also frequently used in the clinic to adjust the imbalanced cellular process. TGF-β1 has been reported to upregulate Cst C secretion from vascular smooth muscle cells, murine embryo cells, cultured differentiated podocytes, 3T3-L1 fibroblasts, and more recently human lung fibroblasts.[13,51,90] Clinically, a significant influence of circulating Cst C levels by TGF-β1 were also observed in patients with thyroid dysfunction, and both Cst C mRNA and protein levels were increased by TGF-β in cultured human hepatoblastoma cells.[91]

Dexamethasone is a potent synthetic member of glucocorticoid class commonly used in the clinic to treat many inflammatory and autoimmune conditions. Interestingly, this steroid drug can drive promoter-mediated upregulation of Cst C gene transcription, which leads to a significant and dose-dependent increase in the Cst C production by up to 80%.[92] Recently, dexamethasone induced secretions of Cst C from human cancer cells were found to be enhanced by co-application of cisplatin and 5-fluorouracil, two agents commonly used in esophageal cancer chemotherapy,[93] adding further factors to the repertoire of Cst C-altering elements.

Physicochemical damages

Rich in cerebrospinal fluid and brain tissue, Cst C is susceptible to physical and chemical insults that may occur in the central nervous system. For example, enhanced Cst C expression was observed in response to all sorts of neurological injuries, including transient forebrain ischemia[94] and seizure.[95] Consistently, the severity of neuronal damage in the CA1 subfield of the hippocampus correlates with enhanced Cst C immunostaining in microglia, the major Cst C-expressing cell type in normal brain tissues.[96] Indeed electrical induction of a status epilepticus causes upregulation of Cst C expression in rat neurons and glia.[97] In addition, persistent environmental toxicants, like dieldrin, and neurotoxin MPP (1-methy1-4phenyl-1,2,3,6-tetrahydropyridine), were also reported to injure dopaminergic neurons and stimulate their secretion of Cst C for microglia activation and neurotoxicity.[98]

Another Cst C-sensitive tissue that is also vulnerable to physical impairment comes from blood vasculature where proteolytic activity of cysteine proteases requires strict regulation by their endogenous inhibitor. Balloon injury was recently reported to increase serum Cst C levels, which correlated with proliferating cell nuclear antigen in smooth muscle cells.[99]

Oxidative stress

Interestingly, the two tissues vulnerable to Cst C levels and damages as mentioned above are also sensitive to the disturbances in their normal redox state. Oxidative stress initiates pathological progression through the production of peroxides and free radicals that damage all components of the cells, including proteins, lipids and DNA. Although the exact mechanisms by which Cst C is possibly involved in the disease development remain to be clarified, oxidative stress was repeatedly reported to upregulate Cst C expression in neurological and cardiovascular systems. For example, oxidative stress causes an increase in Cst C expression in cultured rat primary neurons[19] and cerebral microvascular smooth muscle cells.[100] Moreover, 6-hydroxydopamine induced a temporal and concentration-dependent increase in Cst C secretion from pheochromocytoma cells.[101] The release of Cst C in response to H_2O_2 as one of the cytoprotective, anti-apoptotic factors from the embryonic stem cells were believed to be the mechanism to explain why transplanted embryonic stem cells subsequent to myocardial infarction differentiate into the major cell types in the heart and improve cardiac function.[102] Interestingly, this H_2O_2 induced release of Cst C was not only observed in embryonic stem cells but was also observed later in

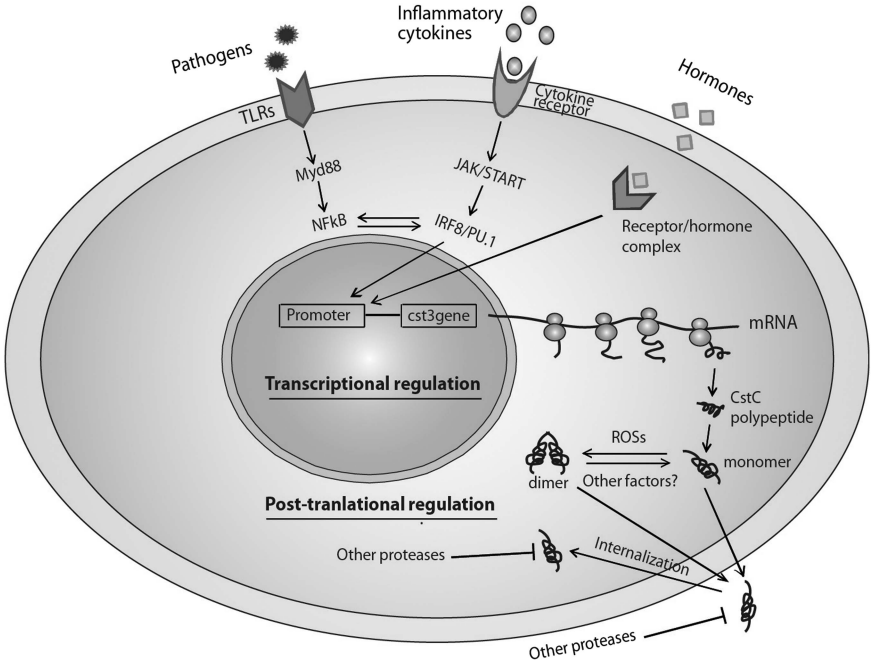

Figure 1 Regulation of cystatin C. Multiple regulatory mechanisms exist to modulate the production and activity of cystatin C inside and outside of cells, at transcriptional and post-translational levels. JAK, Janus kinase; MyD88, myeloid differentiation primary response gene (88); ROSs, reactive oxygen species; STAT, signal transducer and activator of transcription; TLR, Toll-like receptor. A full color version of this figure is available at the *Immunology and Cell Biology* journal online.

cardiomyocytes isolated from rat hearts because coronary artery blocking-induced myocardial ischemia causes an increase in the levels of Cst C protein in the plasma.[103]

Others

Other factors affecting Cst C production include the lung toxicants crystalline silica and arsenic trioxide, which were reported to stimulate Cst C release from rat alveolar macrophages.[104] In cancer patients during malignant progression-like melanoma and colorectal cancer, high serum concentrations of the cysteine proteases cathepsins B and H induced by the tumors stimulate the production of Cst C for counterbalance.[11] In addition, common factors such as cigarette smoking or C-reactive protein, an acute-phase protein in response to inflammation, were also among the lists reported to increase serum Cst C levels independent of renal functions.[9]

MECHANISMS OF CST C REGULATION

The dynamic changes of Cst C caused by different stimuli herald the existence of, and prompt people to search for, the regulatory mechanisms of Cst C to keep this multifunctional and disease-associated protein under check. Like many other proteins, the production of Cst C is subject to both transcriptional and post-translational regulation (Figure 1).

Transcriptional regulation

Although several polymorphisms and sequence variations were detected in the promoter region of *cst3*, the gene coding Cst C,[1,105] it generally shares common features with those of housekeeping genes.[1] Interestingly, the promoter region of murine *cst3* gene was later found to contain a core sequence of the androgen-responsive element and two potential binding sites for activator protein 1,[106] a transcription factor that regulates gene expression in response to a variety of stimuli including cytokines, growth factors, stress and bacterial and viral infection.[107] However, the transcriptional regulation of Cst C was not extensively explored probably due to the ubiquitous expression pattern of the protein. With the increasing recognition of the important roles of Cst C in cardiovascular diseases, the association of this elastolytic cysteine protease inhibitor with human coronary artery disease began to be examined at genetic levels. Two common promoter polymorphisms, a G-to-C substitution at position −82 and a T-to-G substitution at position −78, were found to influence the binding of nuclear factors and affect the basal rate of gene transcription in an allele-specific manner, which are also associated with the plasma concentration of Cst C in healthy individuals and patients with recent myocardial infarction.[36] In another study, the major haplotype −82G/−5G/+4A of the *cst3* gene was found to determine plasma levels of Cst C as the respondents with homozygous genotypes have the highest plasma levels.[108] Collectively, these data suggest that an altered promoter activity of the Cst C gene could be a causal factor for the association between Cst C genotype and plasma Cst C concentration.

Consistent with the potential regulatory mechanisms of human Cst C at genetic levels, we found that Cst C is differentially expressed among mouse cells of the immune system. Specifically, cells of the monocyte/macrophage and DC lineages express it at much higher levels than lymphocytes.[28,89] Mouse DC can be divided into two subsets by their surface expression of CD8α ($CD8^+$ and $CD8^-$ DC). Further analysis of the DC subsets directly isolated from the spleen demonstrated that the $CD8^+$ DC were the major producer of Cst C.[89,109] This was also verified with DCs that had been generated in culture from bone marrow precursors supplemented with Flt3L, in which only the $CD8^+$ DC equivalent, the $CD24^{hi}$ DC subset, contained high levels of Cst C.[110] The differential expression pattern of Cst C among cells of common lineages suggests that its gene could be controlled by cell-specific transcription factors.

This preferential expression pattern of Cst C in monocyte/macrophage and DCs is of clinical relevance in view of the recent reports showing that these cell types are present in tissues where Cst C plays pathological roles such as atherosclerosis and angiopathy. Vascular DCs (aortic DCs), which increase in number in atherosclerotic lesions as disease progresses, were reported to be related to $CD8^+$DC,[111] and the Flt3L-signaling-dependent $CD103^+/CD11b^-$ ($CD8^+$ equivalent) DCs protect against atherosclerosis.[112] Consistently, our analysis of DC purified from aortas indicates that these cells indeed express Cst C (unpublished observation). Similarly, microglia, also called the monocyte/microphage in the brain, is the major cell type that express Cst C in the brain,[96] where deposition of Cst C fibrils produces angiopathy.[64]

To uncover the transcriptional mechanisms underlying the preferential expression of disease-related Cst C in these cells, a novel *cis* element for transcription factors, denoted as IRF (interferon regulatory factor)-Ets composite sequence (IECS), in the promoter region of the *cst3* gene was identified in a reporter assay system employing a self-inactivating retrovirus.[113] This element consists of a core IRF-binding motif for IRF8, and an Ets-binding motif for PU.1, an Ets transcription factor and binding partner that facilitates a more stable binding of IRF8 to chromatin.[114] Notably, both IRF8 and PU.1 are essential for the development of DC subsets,[115,116] of which IRF8 is required for the development of $CD8^+$ $CD103^+$DC,[115] the major producers of Cst C. This could imply that Cst C expression by these cells might be the direct consequence of the presence of IRF8-binding motif in the Cst C promoter region in cells that depend on IRF8 for their development. However, this cannot be the only explanation because IRF8 is also required in plasmacytoid DC development,[117] yet this DC type does not express Cst C.[110] Thus, the molecular mechanisms responsible for this phenomenon remain to be revealed.

To investigate the involvement of these two transcription factors in Cst C expression in primary DC *in vivo*, the physical interaction of IRF8 and PU.1 with the *cis* element IECS was examined by chromatin immunoprecipitation in three different DC subsets freshly isolated from splenic cells.[86] Consistent with their Cst C expression profile ($CD8^+$DC express Cst C, both $CD8^-$DC and plasmacytoid DC do not express Cst C), the binding of IRF8 to the IECS sequence was detected in $CD8^+$DC, but not in $CD8^-$DC and plasmacytoid DC. Interestingly, in the plasmacytoid DC where PU.1 expression is low but IRF8 expression is the same as $CD8^+$DC, the binding of IRF8 to the IECS sequence is still not detected, indicating a quantitative requirement of PU.1 in IRF8 binding to the chromatin, a result consistent with previous findings with fluorescence recovery after filling to mathematical models.[114] Collectively, the chromatin immunoprecipitation data suggest that IRF8 binding to the IECS of the Cst C promoter in the presence of sufficient PU.1 could drive *cst 3* expression. To finally confirm the role of IRF8 in Cst C expression, a $CD8^+$ DC line 1940, where both IRF8 and PU.1 were amply expressed, were transfected with the retrovirus vector LMP encoding shRNAs for *irf8* to silence the gene. When the synthesis of IRF8 was reduced in the cells, that of Cst C, but not MHC I, was also compromised. This causal impact of IRF8 on Cst C production, combined with the diminished Cst C expression in bone marrow (BM)-derived DC from $irf8^{-/-}$ mice compared with wild-type control, strongly suggest that IRF8 is the key transcription factor regulating Cst C expression.[86]

Post-translational regulation

The translational expression of Cst C in quantity does not necessarily lead to a functional protein product. The activity of Cst C can still be further modulated after its transcription. As discussed before, the reactivity of Cst C with its target protease is lost if it dimerizes. Therefore, the factors that cause this conformational change of Cst C by dimerization can be regarded as a way to regulate the activity of this protease inhibitor. It is intriguing to think that Cst C adopts the unreactive dimer conformation as storage form, which quickly monomerizes in response to the stimulation of its target enzymes. This post-translational regulation of Cst C by dimerization was first described in transfected Chinese hamster ovary cells, where Cst C is inactivated during the early part of its trafficking through the secretory pathway and then reactivated prior to secretion.[118] Interestingly, we found that steady-state (*immature*) $CD8^+$ DCs isolated from primary spleens constitutively contain Cst C homo-dimers, which can be separated from monomers by size-exclusion chromatography of cell lysates and their non-reactivity with the Cst C target enzyme papain, supporting the notion that they are domain-swapped dimers. Furthermore, when $CD8^+$ DC underwent maturation by incubation *in vitro*, they no longer produced Cst C dimers.[89] Disappearance of dimer could be the strategy employed by healthy primary cells to process this potentially pathogenic form on their way toward maturation, which might then be disrupted in diseased conditions.

To identify the mechanistic factors leading to Cst C dimerization either as post-translational regulation of its activity, or as amyloid precursor protein, the intracellular accumulation of reactive oxygen species in the immature and mature states were compared. A strong correlation between reactive oxygen species levels and Cst C dimer was observed not only in same cell type at different developmental stages, but also in different cell types at the same developmental stages.[89] Furthermore, artificial enhancement of the intracellular oxidative status resulted in a time-dependent Cst C dimer enrichment, which could be prevented by inhibiting mitochondrial activity, indicating the reactive oxygen species released from mitochondria are responsible for the observed constitutive Cst C dimer formation.[89] Although, the exact process by which oxidative stress exerts the conformational changes of Cst C is not fully understood, a recently published report on recombinant human Cst C stabilized by genetically introduced disulfide linkage demonstrated a disappearance of a dithiothreitol-induced dimer if the concentration of this reducing agent was further increased,[119] suggesting a direct impact of redox environment on the conformational changes of Cst C protein.

Different from type I cystain family members, the type II cystatin Cst C is synthesized with a signal peptide, hence being secreted and consequently found in body fluids.[31] Although its intracellular roles in processing MHCII molecules via lysosomal proteinase in DCs were debatable, the uptake of Cst C in amounts sufficient to affect the activities of intracellular cysteine proteases was described in the eyes,[120] proximal tubule cells[121] and several human cancer cell lines.[122,123] Thus, trafficking inside and outside of the cell membrane of Cst C can regulate or fine tune its division of labor between intracellular compartments and the extracellular matrix. However, the structural determinants on the internalized inhibitor required for efficient uptake were not characterized until recently 12 variants of Cst C with substitutions of selected amino acids were generated.[42] Uptake of Cst C in human breast adenocarcinoma cells is dependent on both charged amino acids of the N-terminal segment and on a hydrophobic amino acid in domains involved in the inhibition of cysteine cathepsins. Furthermore, natural arginine (Arg) residues in positions 24 and 25 are of importance for the uptake process.[42]

Another mechanism for post-translational regulation of Cst C could come from digestion of this cysteine protease inhibitor by proteases of another family. For example, earlier proteomic studies have shown that Cst C is a substrate of matrix metalloproteinase 2 with specific inactivation upon cleavage.[124] In addition, human aspartic

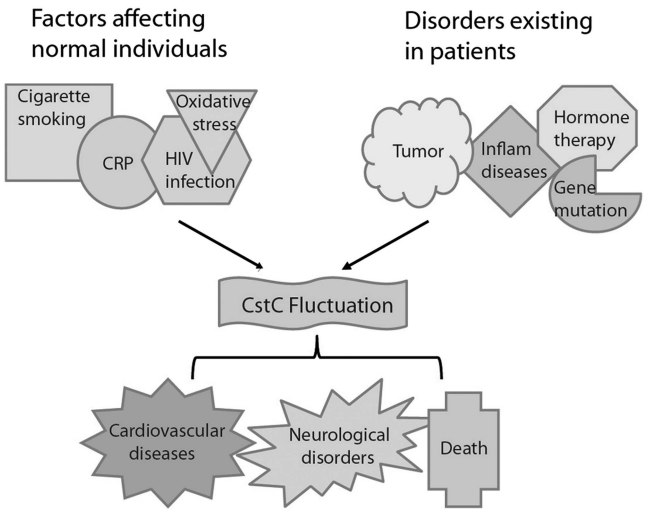

Figure 2 Multiple factors are involved in the progression of cystatin C-related diseases. A scheme summarizing factors leading to the fluctuation of cystatin C levels, which triggles or participates in the pathogenic processes of several diseases. CRP, C-reactive protein. A full color version of this figure is available at the *Immunology and Cell Biology* journal online.

endoproteinase cathepsin D was also able to inactivate human Cst C by cutting hydrophobic amino acid residues into several fragments *in vitro*.[125] Along the same line, the amount of Cst C in the extracellular environment is reduced in the secretome of mouse embryonic fibroblasts stably transfected with human cathepsin D, and the tumor-derived cathepsin D assists breast cancer progression by inhibiting Cst C activity.[126] Importantly, the relevance of this mechanism was also found in other biological process in which Cst C was shown to be a proteolytic target of cathepsin D, affecting the differentiation of DCs from hematopoietic stem cells.[127]

CLINICAL IMPLICATIONS

Given the increasingly discovered roles of Cst C in various pathophysiological processes and identification of its regulatory mechanisms at both transcriptional and post-transcriptional levels, manipulation of Cst C expression either locally or systematically may have many clinical implications. Characterization of the factors that control Cst C expression at the transcriptional level could provide valuable clues for the treatment of pathologies associated with insufficient control of extracellular proteases. For example, signaling molecules that upregulate Cst C expression could be used to promote Cst C secretion in the case that requires slightly higher Cst C concentration to protect neuronal cells from cell death in Alzheimer's disease,[78] or at sites of inflammation in which excessive protease activity causes tissue damage, as has been suggested to occur in atherosclerosis and aortic aneurysm.[13] However, the factors associated with the Cst C dimerization or cytokines that repress Cst C expression could be developed for the treatment of diseases associated with formation of Cst C amyloid. Moreover, identification of the receptors or signaling pathways that initiate the Cst C uptake on the cell surface could be translated to target this potent inhibitor to intracellular cancer-promoting proteolysis via the Cst C internalization.

Since Cst C dimerization loses its activity as a protease inhibitor, which could also be an initial step for the amyloid formation in hereditary cystatin C amyloid angiopathy patients, the development of Cst C dimer/oligomer specific antibodies could be used to make diagnostic screening of hereditary cystatin C amyloid angiopathy family members for early prevention, or selectively remove Cst C dimers from biological fluids containing both dimers and monomers in patients with hereditary cystatin C amyloid angiopathy. In addition, the mechanistic factors affecting the inhibitive activity of Cst C can also be harnessed for therapeutic gains. Drugs to improve the intracellular redox environment by removal of reactive oxygen species and compounds may be beneficial in not only reducing the Cst C amyloidogenesis, but also regulating the elastic proteolysis in the diseased locus as elevated protease activity in the local body fluids is partly responsible for the tissue destruction in the disease associated with Cst C amyloid.[64]

Last but not least, identification of transplantable cellular sources for major Cst C production will also be of great clinical value. Since Cst C is involved in inflammatory diseases, in which immune cells accumulate and play important roles, bone marrow-derived cells are an important cellular source for Cst C manipulation. Along this line, we found that hematopoietic cells contribute significantly to the systematic pools of Cst C.[89] Therefore, bone marrow transplantation would be an applicable approach in clinic to treat patients with Cst C amyloidogenesis or reduction.

CONCLUSION

With the recent identification of regulatory elements in the promoter region of Cst C and increasing reports of factors affecting its production and/or activities, precaution should be taken when Cst C is used as an index of GFR because its blood concentration is subject to changes caused by many factors independent of kidney function. These factors include cigarette smoking, body composition, viral infection, tumor malignancy or gene mutations. Ultimately, the altered Cst C levels or activity could lead to pathological processes in cardiovascular diseases, neurological disorders or even mortality (Figure 2). Such an important disease-associated protein thus should be effectively regulated for therapeutic gains. Further characterization of the signaling pathways leading to Cst C expression will help to develop antagonists and/or synagonists for medical intervention or regulation of the abnormal production and/or activity of this disease-associated enzyme inhibitor. Likewise, identification of the post-translational modifications in the microheterogeneity of the pathogenic species will also assist in understanding the mechanisms or direct factors triggering Cst C dimerization. The knowledge should provide the information for a better understanding of the mysterious mechanisms underlying the diseases associated with Cst C abnormalities.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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