Radiol Oncol 2002; 36(2): 131-43.

review

Natural inhibitors of tumor-associated proteases

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The turnover and remodelling of extracellular matrix (ECM) is an essential part of many normal biological processes including development, morphogenesis, and wound healing. ECM turnover also occurs in severe pathological situations like artherosclerosis, fibrosis, tumor invasion and metastasis. The major proteases involved in this turnover are serine proteases (especially the urokinase-type plasminogen activator/plasmin system), matrix metalloproteases (a family of about 20 zinc-dependent endopeptidases including collagenases, gelatinases, stromelysins, and membrane-type metalloproteases), and cysteine proteases. In vivo, the activity of these proteases is tightly regulated in the extracellular space by zymogen activation and/or controlled inhibition. In the present review, we give an overview on the structure and biochemical properties of important tumor-associated protease inhibitors such as plasminogen activator inhibitor type 1 and type 2 (PAI-1, PAI-2), tissue inhibitors of metalloproteinases (TIMP-1, -2, -3, and -4), and the cysteine protease inhibitor cystatin C. Interestingly, some of these inhibitors of tumor-associated proteases display multiple functions which rather promote than inhibit tumor progression, when the presence of inhibitors in the tumor tissue is not balanced.

Key words: cystein protease; cystatin; matrix metalloproteinase; serine protease; serpin; tissue inhibitor of matrix matalloproteinase

Received 11 March 2002
Accepted 10 April 2002

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This paper was presented at the "2nd Conference on Experimental Tumour Biology", Bovec, Slovenia, March 14-17, 2002.

PAI-1 and PAI-2, natural inhibitors of the urokinase-type plasminogen activator (uPA)

The tumor cell surface-associated urokinase-type plasminogen activator system consists of the serine protease uPA, its receptor uPAR, and the inhibitors PAI-1 and PAI-2. Various normal and cancer cells produce uPA as a single-chain pro-enzyme (pro-uPA) that is proteolytically converted to an active two-chain form, *e.g.* by plasmin or cysteine proteases such as cathepsins B and L.[1] Binding of uPA to uPAR (CD87) focuses the proteolytic activity on the tumor cell surface. In addition to uPAR, tumor cells also express binding sites for plasmin(ogen). uPAR-bound uPA efficiently converts tumor cell-associated plasminogen into plasmin, an active serine protease with broad substrate specificity. Plasmin degrades a variety of components of the extracellular matrix (*e.g.* fibrin, fibronectin, or laminin) and activates several matrix metalloproteases that additionally break down certain macromolecules of the extracellular matrix such as fiber-forming collagens and/or the basement membrane protein collagen IV.[2,3]

The uPA/uPAR system is under the control of the plasminogen activator inhibitors type 1 (PAI-1) and type 2 (PAI-2) both belonging to the serine protease inhibitor super-family (serpins) and sharing 55 % sequence homology (amino acid identity: 33 %). Their structural similarity was shown by X-ray crystal structures of active mutants of PAI-1 and PAI-2 (Figure 1A, B).[4,5] These two inhibitors interact with uPA and the other plasminogen activator, tPA (tissue-type plasminogen activator), forming 1:1 stoichiometric complexes with the respective target protease. tPA, in contrast to uPA, does not bind to a high-affinity receptor on tumor cell surfaces and, therefore, does not promote tumor cell-associated pericellular proteolysis. Whereas PAI-1 recognizes and inhibits all active forms of the proteases (two-chain uPA as well as single- and two-chain tPA), PAI-2 only acts as an inhibitor for the two-chain forms of uPA and tPA.[6] For inhibition, the surface-exposed reactive center loop (RCL) of PAI-1 or PAI-2 interacts with the reactive site of the target protease. Initially, the P1-P1′-bond of the inhibitor (R346-M347 in the case of PAI-1; R380-T381 in the case of PAI-2) is cleaved and a covalent bond between the hydroxyl-group of the catalytic serine residue of the protease and the carboxyl-group of the P1-residue of the RCL of the serpin is formed (acyl-enzyme intermediate). Upon cleavage of the P1-P1′-bond, the RCL is rapidly inserted into the central β-sheet A as additional β-strand 4A, which leads to the translocation (> 70 Å) of the protease across the plane of β-sheet A of the serpin and the formation of a stable enzyme/inhibitor complex. The structure of PAI-1 or PAI-2 in complex with a target protease has not been solved yet, however, the X-ray crystal structure of another serpin-protease complex (trypsin/antitrypsin) has recently been published.[7] The experimental data confirm the theoretical model for the inhibition of serine proteases by serpins.[8,9] In the trypsin/antitrypsin complex, the structure of the hyperstable serpin is hardly changed, whereas the active site of the protease is massively disordered, which prevents the release of the protease from the complex.[7]

PAI-1 is synthesized as a 402 aa-protein (inclusive an N-terminal signal peptide) and secreted by the cell in a glycosylated form. The mature protein (379 aa; M_r: ≈ 50,000) contains no cysteines and, therefore, no disulfide bridges. Active PAI-1 is meta-stable and spontaneously converts to a latent form (which does not inhibit its target serine proteases) by inserting a major part of its RCL into the central β-sheet A (Figure 1C). The biologically active conformation of PAI-1 (half-life: ≈ two hours) is stabilized by vitronectin (Vn), an extracellular matrix (ECM) and plasma protein (half life of PAI-1 bound to Vn: ≈ four hours).[10] Upon binding of Vn to active PAI-1

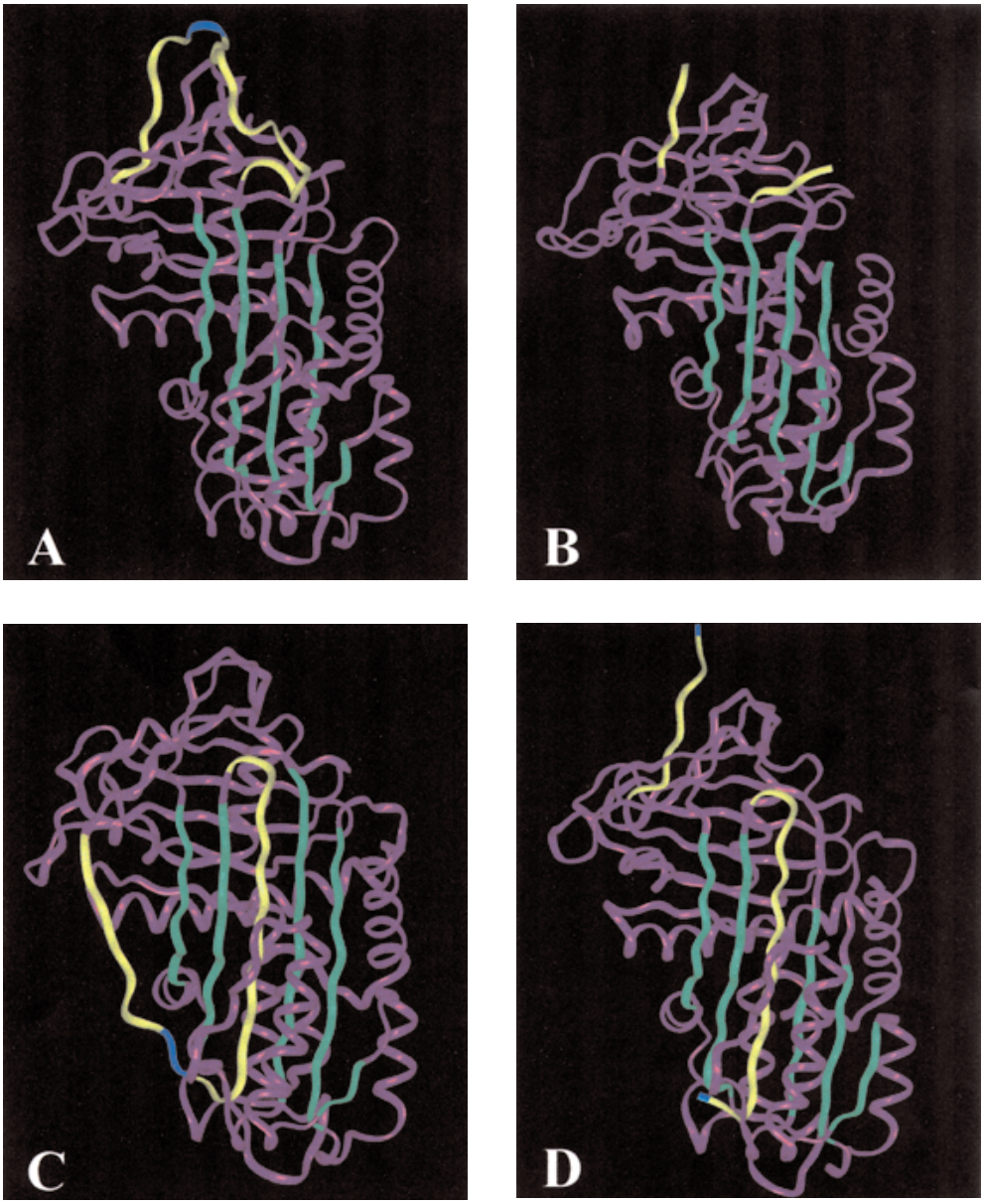

Figure 1. The serpins PAI-1 and PAI-2
Ribbon representation of the tertiary structure of PAI-1 and PAI-2: **(A)** active conformation of PAI-1 (pdb-code: 1B3K), **(B)** active conformation of PAI-2 (pdb-code: 1BY7), **(C)** latent conformation of PAI-1(pdb-code: 1DVN), and **(D)** substrate cleaved form of PAI-1 (pdb-code: 9PAI). The central β-sheet is colored green, the reactive center loop (RCL) is yellow and the localization of the P1 and P1′ residues are blue. In the active conformation of PAI-1 **(A)**, the RCL represents a very flexible loop which is interlaced as an additional β-strand in the latent conformation **(C)**, while in the substrate cleaved form the essential residues P1 and P1′ of the RCL are pulled far apart **(D)**. Because of its flexibility, the localisation of the RCL of PAI-2 was not clearly defined in the X-ray structure and is therefore not depicted in **(B)**. This is also true for several other parts of the molecule. The figures were drawn on the coordinates from the Brookhaven Protein Database using the computer program INSIGHT II (Molecular Simulations Inc.).

structural changes in PAI-1 are induced, providing it with the inhibitory properties against the serine proteases other than uPA and tPA, namely thrombin and activated protein C.[11,12] Recently, alpha(1)-acid glycoprotein has been shown to interact with PAI-1 as well and to stabilize the active form of this inhibitor.[13] In addition to Vn and alpha(1)-acid glycoprotein, PAI-1 interacts with heparin, fibrin, and – when present as a PAI-1/uPA complex – with members of the lipoprotein receptor-related protein (LRP) receptor family.[6] Under certain conditions, when the distortion of the active site of the protease in complex with the serpin cannot keep pace with ester bond hydrolysis, PAI-1 can also be cleaved in a substrate-like manner, and the inhibitor is released from the active protease as the so-called RCL-cleaved form of PAI-1 (Figure D).[14,15]

PAI-2 lacks a cleavable signal peptide and is mainly present intracellularly in a non-glycosylated form (415 aa; M_r: ≈ 47,000). Only a small amount of PAI-2 (≈ 20%) is glycosylated and secreted (M_r: ≈ 55,000). PAI-2 spontaneously forms polymers, very likely by a loop-sheet polymerisation mechanism, in which the RCL of one molecule inserts as an additional β-strand into the central β-sheet of another molecule.[16] The mainly intracellular location of PAI-2 developed a hypothesis that this serpin has some other functions in addition to the inhibition of plasmin generation (which occurs extracellularly). In fact, PAI-2 has been shown to inhibit apoptosis.[17] Furthermore, PAI-2 may function as an antiviral agent and be of relevance in Alzheimer's disease and in some inflammatory reactions.[16,18] The inhibitory effect of PAI-2 depends on both the active site and interhelical loop between helices C and D. This loop has been implicated in transglutaminase-catalyzed cross-linking of PAI-2 to cell membranes.[17] Besides the target proteases uPA and tPA (and transglutaminases), no further intra- or extracellular interaction partners of PAI-2 have been identified so far. In addition to PAI-1 and PAI-2, there are other serpins, *e.g.* proteinase nexin-1, protein C inhibitor, and maspin (a serpin with tumor suppressive activity), which are also capable to inhibit uPA (and tPA) under physiological conditions.[3,19]

PAI-1 and PAI-2 in cancer

In a variety of malignancies such as breast, ovarian, esophageal, gastric, colorectal or hepatocellular cancer, a strong clinical prognostic impact has been attributed to components of the uPA-system, especially PAI-1 and uPA that are statistically independent factors with the capacity to predict the probability of disease-free and/or overall survival.[1,20-23] In general, elevated tumor antigen levels of PAI-1 and/or uPA are associated with poor disease outcome and are conductive to tumor cell spread and metastases. The clinical finding that the uPA inhibitor PAI-1 does not exert a protective function but is an indicator of bad prognosis for cancer patients appears rather striking at first sight. However, additional functions of PAI-1 have been described, which strongly suggest an involvement of PAI-1 in the tumor promoting processes, especially in the modulation of tumor cell attachment or migration and in angiogenesis.[24,25] As the binding sites of PAI-1 and uPAR on the ECM protein Vn overlap, PAI-1 is able to regulate uPAR-mediated cell adhesion by competing with uPAR for binding to Vn. Furthermore, PAI-1/Vn-interaction also affects integrin-mediated cell adhesion to Vn by sterically blocking integrin binding to the RGD (Arg-Gly-Asp) sequence which is immediately adjacent to the PAI-1 binding site on Vn.[26,27] Malignant murine keratinocytes, transplanted into PAI-1-deficient mice, did not invade the surrounding tissue (local invasion). Additionally, the PAI-1-deficient hosts failed to vascularize the implanted tumor cells. Upon intravenous injection of an ade-

noviral vector expressing human PAI-1 in these tumor-bearing mice, tumor cell invasion and associated angiogenesis were restored.[28] Some recently published data report of plasmin involvement in the assembly of new tumor vessels and indicate that PAI-1 is essential for controlling excessive plasmin proteolysis which would otherwise prevent the formation of these vessels.[25,29] The PAI-1/Vn-interaction may also play a part in tumor neovascularization.[29]

In contrast to the consistent association of high tumor tissue concentrations of PAI-1 (and uPA and uPAR) with poor prognosis, various studies analyzing the prognostic impact of PAI-2 have shown different associations between the PAI-2 levels in tumor tissue and patient survival. On the one hand, high antigen levels of PAI-2 in tumor tissue have been associated with good prognosis in patients with breast cancer, small-cell lung cancer and ovarian cancer, but on the other, with a poor prognosis in colorectal and endometrial cancer.[30,31]

Biochemical properties of the TIMPs, the inhibitors of matrix metalloproteases

In addition to the uPA/plasmin system, there is compelling evidence that matrix metalloproteases (MMPs) also act as key players in the events that underlie tumor dissemination.[32] Tumor and stromal cells produce soluble and cell-surface anchored MMPs, which mediate ECM degradation, release of sequestered latent growth and angiogenic factors, and activation of latent growth factors. The proteolytic activity of MMPs is controlled by the so-called TIMPs.

Currently four members of the TIMP-family (TIMP-1, -2, -3 and -4) are known and characterized.[33] These are small proteins with a molecular weight between 21 kDa and 28 kDa and are secreted by many different cell types. They share common structural features including an N-terminal and a C-terminal subdomain, each stabilized by 3 disulfide bonds. Each TIMP has 12 conserved cysteine residues, contributing to the secondary structure and their ability to inhibit MMPs.[34,35] TIMP-1 is glycosylated (8-9 kDa), TIMP-3 can contain sugar components up to 7 kDa;[36] whereas TIMP-2 and TIMP-4 are non-glycosylated.

The N-terminal domain of the molecules harbors the inhibitory activity which forms a tight 1:1 non-covalent complex with the catalytic center of active MMPs (Figure 2). Binding to latent MMPs occurs in a 1:1 stoichiometry at the C-terminal region of individual MMPs.[37] Recombinant truncated TIMPs containing only the N-terminal domain retain most of their inhibitory activity towards

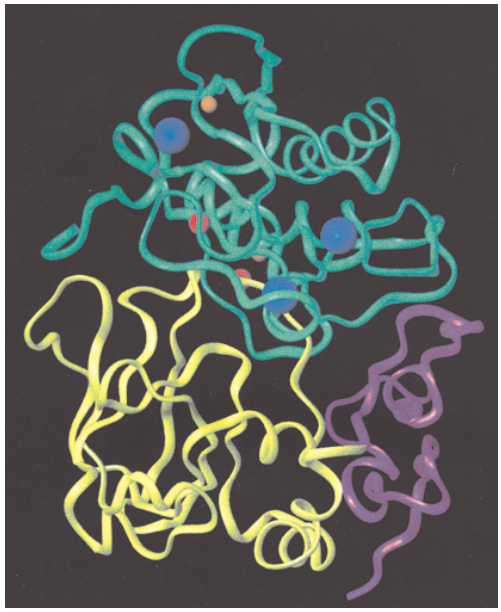

Figure 2. The TIMP-1/MMP-3 complex.
Ribbon representation of the TIMP-1/MMP-3 complex structure (pdb-code: 1UEA): the N-terminal domain of TIMP-1 (residues 2-126) is colored yellow, the C-terminal domain in magenta; MMP-3 is green, catalytic zinc is red, calcium is blue and selenium orange. Only the N-terminal domain of the TIMP-1 molecule performs interactions to the MMP-3 protein in the complex. The figures were drawn on the coordinates from the Brookhaven Protein Database using the computer program INSIGHT II (Molecular Simulations Inc.)

MMPs.[38] The four different TIMPs bind to most subtypes of latent and active forms of MMPs with only minor differences in their inhibitory potential. Variable affinities were elucidated for TIMP-1 and TIMP-2, the former binding to the latent form of MMP-9 with a higher affinity than the latter, while the converse relationship was found for binding to latent MMP-2.[39] High levels of TIMP-2 or -3, but not of TIMP-1 inhibit the activity of MT1-MMP, thereby preventing the latent MMP-2 activation.[40]

The C-terminal domain of the TIMPs is more variable and is involved in the interaction with pro-MMPs.[41,42] Furthermore, this domain might be responsible for the additional biological functions of the TIMPs in proliferation, angiogenesis, and apoptosis.[43,44] These effects are independent of the inhibitory function of TIMPs; however, the mechanisms of these actions are not understood yet.

TIMP-3 is unique because it is unsoluble and tightly bound to the extracellular matrix by its C-terminal domain. Furthermore, TIMP-3 is correlated with the hereditary disease Sorsby`s fundus dystrophy that is caused by a single base pair exchange in the sequence coding for the C-terminal domain.[45]

Modulation of tumor growth and metastasis by TIMP expression in the tumor environment

Recent clinical studies have stated TIMP-1 and TIMP-2 to be rather tumor-promoting molecules, as they were found to be significantly overexpressed in patients with poor prognosis.[46,47] However, it is important to note that, in these studies, the MMP/TIMP ratio was not determined, while the evaluation of either TIMP or MMP expression alone is likely not sufficient for prognostication of malignancies. It is generally accepted that the net proteolytic activity in the tissue is responsible for tumor cell invasion-promoting ECM turnover. Consequently, (gene-) therapeutic intervention by overexpression of TIMPs in the tumor microenvironment is supposed to inhibit ECM degradation and metastasis.[48] The MMPs, the target molecules for the TIMPs, are proteolytically active in the extracellular space. Therefore, in a therapeutic approach, it is not necessary that all tumor cells are transduced by gene therapy vehicles to express elevated levels of TIMPs. It is sufficient that host cells, located at the interface surrounding the primary tumor or the invading cells in the target organ of metastasis, overexpress and secrete TIMPs. The proteolytic balance in the tumor-host environment could thus be shifted in favor of blocking proteolysis, resulting in the inhibition of tumor invasion and metastasis. TIMP-1 transgenic mice have been used to elucidate the feasibility of such an approach and to assess the protective potential of host TIMP-1 on primary tumor growth and metastasis. In the first of these studies, the crossing of transgenic mice that constitutively overexpress TIMP-1 in the liver with transgenics expressing SV40 T antigen, which develop hepatocellular carcinoma, resulted in inhibiting the tumor initiation, growth, and angiogenesis.[49] In another approach, two transgenic mouse lines were used, one overexpressing TIMP-1 (TIMP-1^{high}), and the other expressing the antisense TIMP-1 RNA, leading to TIMP-1 reduction in the tissue (TIMP-1^{low}).[50] TIMP-1 overexpression (TIMP-1^{high}) inhibited tumor growth and spontaneous metastasis of an aggressive T-cell lymphoma, thereby prolonging the survival of mice. Opposite effects occurred in TIMP-1^{low} mice: experimental metastasis assays demonstrated that TIMP-1-compromised livers in TIMP-1^{low} mice showed at least a doubling of metastatic foci and numerous additional micrometastases, indicative of increased host susceptibility.[51] In another experimental setting, experimental metastasis of a fibrosarcoma in the brain could significantly be inhibited in transgenic mice overexpressing TIMP-1.[50] Similar studi-

es with transgenics for TIMP-2, -3, and -4 have not been reported so far. The encouraging results with TIMP transgenic mice have stimulated preclinical gene therapy experiments in mice. Employing adenoviral vectors for TIMP-2 gene transfer to the liver of mice prevented the growth of colorectal metastasis in this organ.[52] Recently, the protection of the host environment was provided by the dramatic overexpression of TIMP-1 due to adenoviral gene transfer, inhibiting the growth of experimental liver metastasis of the T-cell lymphoma (employed in the studies with the transgenic mice mentioned above) and colorectal carcinoma.[53] The systemic increase of TIMP-4 due to intramuscular gene delivery resulted in the inhibition of Wilm's tumor growth,[54] but induced mammary tumorigenesis, most likely due to the anti-apoptotic features of TIMP-4.[55] Gene therapy with vectors encoding TIMP-1 or TIMP-3 to protect the host environment from metastasis has not yet been documented.

The vast literature on the genetic alteration of tumor cells themselves with TIMPs has revealed conflicting data on the usefulness of natural TIMPs in the direct genetic modification of tumor cells. However, these studies indicate that genetic engineering of TIMPs devoid of their additional biological functions (*e.g.* growth factor activity) to increase anti-tumor specificity, might be a useful therapeutic approach.[33]

Cystatins, the natural inhibitors of cysteine proteases

Cystatins comprise single-chain inhibitory proteins that reversibly inhibit the proteolytic activity of cysteine proteases, which are widely distributed in the human body.[56-59] Three types of cystatins are present in vertebrates: type-1 cystatins that are synthesized without a signal peptide and generally present in the cell (cystatin A and B, also named stefin A and B); the secretory type-2 single-domain cystatins (C, D, M/E, F, S, SN, SA) and type-3 multi-domain cystatins (high and low molecular weight kininogens). The type-1 cystatins (approx. 100 aa; M_r: ≈ 11- 12,000) lack both disulfide bridges and carbohydrate groups. Type-2 cystatins (*e.g.* chicken cystatin and human cystatin C) are molecules of about 120 aa (M_r: ≈ 13-14,000) and are characterized by two intrachain disulfide bonds located towards the C-terminus. With the exception of the rat cystatin C, type-2 cystatins are non-glycosylated.[60] Type-3 cystatins encompass three type-2 cystatin-like domains that most probably arose by gene duplications.[61] They contain additional disulfide bonds and are glycosylated.

The secondary structures of chicken cystatin, human cystatin C, and the type-1 cystatins are very similar. A cystatin molecule consists of an N-terminal straight five-turn α-helix ((1) and a five-stranded antiparallel β-pleated sheet (β1 N-terminal and β2-β5 C-terminal), twisted and wrapped around the α-helix.[62] In the case of human cystatin C, no evidence was found for an α-helical conformation of the region unique to the type-2 cystatins (aa T71-L91) that was found in chicken cystatin.[63]

As further demonstrated by crystallographic studies, human cystatin C forms very tight symmetric dimers by a mechanism called three-dimensional domain swapping.[64] Furthermore, higher aggregates may arise through this mechanism in an open-ended way, in which partially unfolded molecules are linked into infinite chains, also found in the brain arteries of elderly people with amyloid angiopathy. An even more severe disease is associated with the L68Q mutant of human cystatin C that destabilizes the monomers and increases the stability of the partially unfolded intermediate causing massive amyloidosis, cerebral hemorrhage, and death in young adults.[63]

Cystatins form equimolar, tight and reversible complexes with papain-like cysteine

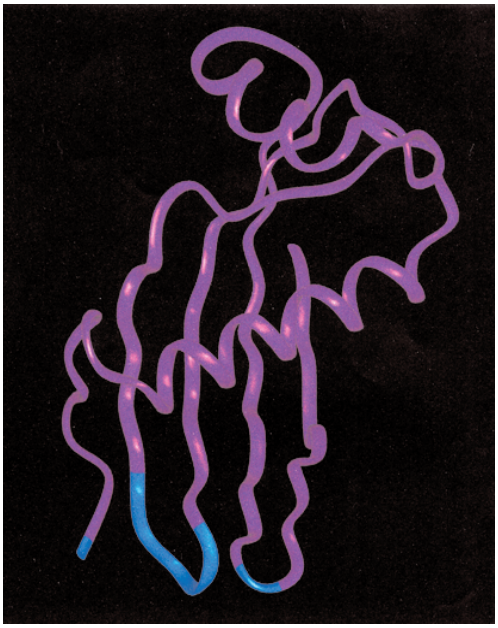

Figure 3. Chicken cystatin.
Ribbon representation of the structure of chicken cystatin (pdb-code: 1CEW). The residues essential for the inhibition of cysteine proteases are distributed on three different adjacent loops and are colored blue. The figures were drawn on the coordinates from the Brookhaven Protein Database using the computer program INSIGHT II (Molecular Simulations Inc.)

proteases.[65] There are three well-conserved regions in the cystatin superfamily that have been implicated in cysteine protease inhibition (Figure 3). These regions are (i) a region near the N-terminus, (ii) a first hairpin loop containing the highly conserved sequence Gln-Xaa-Val-Xaa-Gly, and (iii) a second hairpin loop containing a Pro-Trp pair.[62,66] All three regions contain several hydrophobic residues, indicating that hydrophobic interactions play an important role in the interaction of cystatins with target molecules. Based on structural theory, these three regions penetrate the active site of the enzyme in such a way that the papain active site Cys25 residue is blocked.[62] An additional reactive site in the loop between the α-helix and the first strand of the main β-pleated sheet with its Asn39 residue was detected and shown to be responsible for the inhibition of mammalian legumin by some cystatins.[67]

The role of cystatins in cancer progression

In mammals, cystatins are found in relatively high concentration in biological fluids such as seminal plasma, cerebrospinal fluid, plasma, saliva, and urine. Cystatins A, B, and C are present in high molar concentration in various cells and tissues,[66,68] whereas cystatins D, S, SN, and SA are almost limited to saliva, tears, and seminal plasma.[59,69] Kininogens are major plasma proteins, involved in the tonus regulation of blood vessels and coagulation in addition to their function as cysteine endopeptidase inhibitors.[65]

A large number of normal and pathophysiological processes are controlled by balancing cysteine proteases and their inhibitors. Uncontrolled proteolysis by human cysteine proteases can cause irreversible damage such as inflammatory diseases, neurological disorders, infection, and tumor metastases.[70-74] Cathepsin B, H, and L, primarily lysosomal cysteine proteases, are also important matrix proteases that are involved, together with plasminogen activator and metalloproteases, in cancer invasion by degrading extracellular matrix components.[75-78] Cystatin C is the strongest inhibitor of cysteine proteases, it was therefore most frequently investigated in tumor invasion and metastasis. Tumor-associated expression of cystatin C was at first detected in the ascitic fluid from patients with ovarian cancer.[79] Cystatin C is also increased in the blood of patients with breast cancer,[80] fibrosarcoma,[81] melanoma,[71] colorectal carcinoma,[73] and lung cancer.[82] In these studies, it was reported that the cystatin C content in melanoma, colorectal, and lung cancer patients is associated with the progression of the malignant disease. Furthermore, cathepsin B/cystatin C complexes were found to be less abundant in the

blood of patients with malignant tumors than in healthy controls indicating an imbalance between cysteine proteases and cystatin C in cancer cells.[83] In comparison to normal tissue, a significant decrease of cystatin activity in breast carcinomas compared to normal tissue was noted[80] also in the cerebrospinal fluid and blood from patients with brain tumors.[84] An elevated concentration of the latent (inactive) fraction of cystatins was determined in the blood of patients with head and neck cancer and in the urine of patients with colorectal cancer.[85,86] The significant decrease of the inhibitory activity of cystatins in biological fluids in cancer patients may be taken as a further support to the assumption of an involvement of cysteine proteases in tumor progression and metastasis.[75]

Cystatin C activity is, in fact, correlated to the malignant phenotype as shown by *in vitro* and *in vivo* tumor model systems. Transfection of cystatin C cDNA into B16 melanoma cells led to an inhibition of tumor cell invasion through an artificial matrix barrier *in vitro* and to a significant reduction of the number of lung metastases after the injection into the tail vein of nude mice.[87,88] The inhibitory effect of cystatin C on tumor cell invasion was also demonstrated in *in vitro* Matrigel assays using transfected murine SCC-VII squamous carcinoma cells,[89] ras-transformed breast epithelial cells,[90] human fibrosarcoma, and colon carcinoma cell lines.[81] It is worth to mention that, in cystatin C-deficient mice, a reduction of lung colonization of mouse melanoma cells (expressing cystatin C) was observed.[91] This indicates that an excessively high local cysteine protease activity may inhibit metastatic spread to some tissues. Since other proteases including uPA and matrix metalloproteases are also key molecules in tumor cell metastasis, it is an interesting strategy to modulate the activities of cysteine and other proteases simultaneously.[92]

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