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Cystatins and cancer

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1. ABSTRACT

Cystatins are natural cysteine protease inhibitors which belong to a superfamily of proteins with wide occurrence in tissues. The cystatins have been shown to play multiple roles in normal and disease processes. In many different cancers the cathepsins, enzymes inhibited by cystatins, are elevated and participate in tumor growth and invasion. The levels of the cystatins can vary quite widely in different cancers. Recent studies have shown cystatins can block invasion or metastasis of different cancers in experimental systems. Insights into cystatin roles in cancer have provided links to tumor development, angiogenesis, and tumor cell death in this devastating disease.

2. INTRODUCTION

The cystatins are a superfamily of cysteine protease (C1 family, cathepsin) inhibitors. These inhibitors are proteins which carry out reversible, tight binding complexes with certain papain-related cysteine proteases in tissues and biological fluids. While a primary role of the cystatins is to keep excessive cysteine protease activity in check, new roles for the cystatins have also been discovered (1). Cystatins have been linked to multiple diseases and pathological states such as arthritis, atherosclerosis, amyloidosis, and cancer (see accompanying articles by Bengtsson *et al.* and Nagai *et al.*). The role of cystatins in cancer has been an active area of investigation, particularly since the late 1980s. As

important regulators of cathepsin activity, cystatins in cancer have progressed alongside research on the cathepsins. Cathepsins are primarily endoproteases located in the endocytic pathway and concentrated within lysosomes, but extracellular forms are also found in most cancers (2). Thus, much of the critical foundation for the understanding of cystatin function in cancer has been achieved through analysis of cystatin and cathepsin levels in tumor samples and in a wide variety of cancer cell types. Because of the complexity of cancer development and the sheer diversity of cancer types, knowledge of cystatin involvement in cancer is somewhat fragmentary. Therefore, an overview of cystatin involvement in cancer will be presented here with emphasis on recent advances.

The primary role of cystatins as protease inhibitors is to limit excess cysteine protease activity released from lysosomes or produced during inflammation (3). As cysteine proteases play roles in tumor development, growth, and, metastasis, the cystatins should be instrumental in helping regulate these processes (4). Information gained from research on the role of cystatins in tumor biology is poised to expand potential targets for effective anticancer agents. In recent investigations, the cystatins have been shown to have new and unexpected roles, such as participation in immune responses and neuronal differentiation (5, 6). Undoubtedly, these new roles will continue to expand our understanding of key pathological processes, including cancer (see Keppler for an excellent earlier review of cystatins and cancer (1)).

As type I and II cystatins are the most studied in cancer, the focus of this review will be on these two types. Other cystatins and cystatin-related proteins are reviewed elsewhere (7, 8). The type I cystatins (stefins) are about 100 amino acids in length, do not possess disulfide bonds, and are found primarily intracellularly. Major species of type I cystatins are cystatins A and B, commonly referred to as stefins A and B. Stefin A has a relatively restricted expression within skin and certain white blood cells. Stefin B has a broad distribution across cell types. The type II cystatins is a family of proteins which currently has 14 members. They are about 120 amino acids in length, have two intra-chain disulfide bonds, and are primarily secreted proteins. Several body fluids, such as spinal fluid and semen, have high levels of type II cystatins (9). Interestingly, genes encoding type II cystatins are found clustered in a specific region on human chromosome 20 (10). Details of the interactions between type I and II cystatins and cysteine proteases have been derived from x-ray crystallographic analysis of inhibitor-protease complexes (11, 12).

3. CYSTATIN LEVELS IN CANCER.

Early studies on the cystatins in cancer focused on the levels of these inhibitors in relation to cathepsins B and L. A general theme that emerged was that the cathepsin to cystatin ratio increased in most tumor types compared to normal tissues, particularly for advanced cancers (13-16). (Increases in cathepsin levels have been described for most invasive cancer types (4)). In addition, secreted and cell membrane bound forms of tumor cathepsins allow for extracellular functions in cancers (17, 18). Therefore, the cathepsins make an important contribution to cancer cell invasion and other aspects of tumor development. In some cases, besides an increase in the cathepsin levels, cancers may also display a decrease in cystatin expression. Upwards of 50% of cancers may show decreased expression of cystatin C and perhaps other cystatins (19). There does not seem to be a general rule for predicting cystatin expression levels when different cancer types are compared. Thus, it is not surprising that tumor cystatin levels have wide variations, and may be of secondary importance to increased cathepsin levels during tumor progression. The relative importance of the cathepsins and cystatins to tumor development may also differ between cancer types. Although the cystatins may, in some cases, behave as a type of 'tumor suppressor', the validity of this concept is still being tested. Rapid progress is being made on the role of cathepsins in tumor progression (see section 4) which will lead to new studies on the cystatins' roles in cancer. Molecular genetics studies and gene array analysis for tumor cystatins and cathepsins will undoubtedly provide a clearer picture in the near future.

3.1. Type I cystatins: stefins A and B

Some epithelial-type cancers have been found to have decreased stefin A expression which correlates with decreased patient survival (20). Early evidence indicated that stefin A levels could be decreased at the protein and transcriptional levels during tumor progression (21). Decreases in stefins A and B were also noted in breast cancer cell lines of increasing invasiveness (22). Stefin A immunostaining was found in benign but not malignant meningiomas (23, 24). This same finding extends to glioblastoma where the invasive capability of tumors can be ascertained by cystatin markers (25). Stefin A immunologic staining was also markedly reduced in pituitary adenomas while cathepsin levels were frequently increased (24). Low stefin A levels correlated with poor patient survival in head and neck cancer and this study deserves follow-up (26). Lower protein and message levels of stefin B were noted in atypical versus benign meningiomas (27). Interestingly, both cathepsins B and L were elevated at the protein but not the message level in atypical meningiomas, suggesting translational control of cysteine protease expression. Diagnostic markers for meningiomas under investigation are a combination of stefin B and cathepsins B and L. Microarray analysis has identified stefin B as a down-regulated gene in melanoma, particularly in lymph node metastatic melanoma cells (28). Through differential expression analysis of genes Shiraishi *et al.* showed stefin B was downregulated in human esophageal carcinoma and this change was related to lymph node-metastasis (29). In general, lower expression of stefins A and/or B are found in aggressive tumor types.

In a recent study, stefin A-positive breast cancer patients were much less likely to develop distant metastasis (30). In general, increases in primary tumor stefin levels seem to correlate with a more favorable prognosis. Small cell lung cancer patients showed levels of stefin A and B

that were higher in tumor than in normal tissue (31). Patients with higher stefin A and B levels had a better prognosis for small cell lung carcinoma. Stefins A and B were also elevated in NSCLC (non-small cell lung cancer) tumor cells compared to normal lung tissues (32). Here too, patients with elevated tumor stefins exhibited a more favorable prognosis. Perhaps because of better proteolytic control in tumor tissues, patients with higher stefin A and B levels tend to display better survival, although this point has not been clearly established. In some cancers elevation of cystatins in the primary tumor correlates with a more favorable prognosis.

Unfortunately, some tumor types have also been shown to have elevated stefin A and or B levels which correlate with poor survival outcomes (33, 34). Elevated sera levels of stefin B (and cystatin C) are found to be prognostic of poor survival for colorectal cancer patients (35). In these cases cystatin levels appear to coincide with increased tumorigenicity and not simply invasiveness of the cancer cells. Paradoxical cystatin levels in different cancers need to be explored more fully in regard to patient outcomes.

3.2. Type II cystatins

For certain cancer types, it has been reported that tumor associated cathepsin levels could be used as prognostic indicators for cancer classification (20, 36). Coupling cystatin levels to cathepsin levels usually strengthens the prognostic results. Nakabayashi *et al.* showed that cystatin C protein and message levels were decreased in high grade glioma tumor masses (14). Cathepsin B was also overexpressed such that coupling information on cathepsin and cystatin levels might be a more useful prognostic indicator for glioma. Work by Nagai *et al.* has also found that, in leptomeningial metastasis, lower cystatin C levels were accompanied by elevated cysteine protease activity (37). Nishikawa *et al.* studied ovarian cancers and found cystatin C levels significantly higher in benign than in malignant cases (16). Further, addition of purified cystatin C to invasive ovarian cancer cells was found to block *in vitro* invasion. For many cancer types a clear increase in cathepsin to cystatin ratio is found for cystatin C.

Changes in cystatin levels during tumor progression appears to be more complicated. The relation between cystatin expression in benign and cancerous prostate tissues was conducted (38). Early stages of prostate cancer were found to have increased levels of cystatin C whereas later stages had decreased levels. It was speculated that cystatin C may be protective at certain stages of prostate tumor progression, perhaps regulating peptide processing events. However, cystatin C levels at later stages of prostate cancer must be examined relative to the cysteine proteases so that a more complete picture may be obtained for prostate cancer progression. Another study showed no change in cystatin C during colorectal cancer progression when tumors from different stages were examined (39). Higher levels of cathepsin B were noted at early and late stages (Dukes A and D), suggesting cathepsin B plays an important role in early local invasion and metastasis. Heavy immunological staining of tumor stromal cells, however, indicated cathepsin B plays a more complicated role in tumor progression. These studies further suggest that the ratio of cysteine protease to cystatin is more important than absolute levels. Zore *et al.* showed a decrease in cathepsin-cystatin C complexes with an increase in stage of colorectal cancers (40). Decreases in cystatin binding to cathepsins could involve some type of protein modification, such as glycosylation, but the details are lacking.

The case for measurement of cystatin C levels as a prognostic factor for cancer is problematical. Generally, an inverse correlation between cystatin C levels and tumor grade has been noted (41). In contrast, late stage cancer patients often have enhanced serum levels of cystatin C (26). In patients with metastatic melanoma the average serum level of cystatin C was found to be higher than normal (470 vs 320 ng/ml) (35). The reason for this increase could have several causes including increased tumor mass. Since cystatin C is freely filtered through the kidneys, kidney function should also be measured in future studies to rule out this potential cause of cystatin elevation (42). Cystatin C measurements, unaccompanied by other cancer-related markers, cannot be recommended for prognosis.

Cystatin F (alternatively known as leukocystatin or CMAP) shows a rather restricted expression pattern, being confined to certain lymphoid cell types and organs (43). In contrast to cystatin C, increased cystatin F has been found for several murine cancer types (44). High cystatin F levels correlated with liver metastasis of colorectal cancer. Little or no expression of cystatin F was found in the primary tumor (45). So far, no mechanism has been put forward for elevated cystatin F and increased liver metastasis. Possible mechanisms include tumor cell protection from cysteine proteases during intravasation, antagonism of other cystatins, or protection from apoptosis of the metastatic tumor cell. This finding certainly suggests caution must be used before general statements are made for cystatin roles in cancer.

Cystatin E/M is expressed in normal tissues, but expression is lost in most late stage/metastatic breast cancers (46). Interestingly, increased cystatin M expression was shown to decrease tumor cell invasion, proliferation, and adhesion to endothelial cells (47). Cystatin M silencing with an RNAi approach in an oral cancer cell line not only increased cell invasion and motility but also increased cell proliferation by an unknown mechanism (48). In contrast, through laser capture microdissection of breast cancer cells, cystatins M (and C) correlated positively with tumor size but not with metastatic ability (49). Vigneswaran *et al.* also described elevated cystatin M in metastatic squamous cell carcinomas (50). It was proposed elevated cystatin M might protect advanced cancers against cathepsin B-mediated cell death. These results are at odds with cystatin M acting as a tumor suppressor, but larger studies may clarify differences between tumor types.

4. CYSTATINS AND CANCER INVASION-METASTASIS

Cathepsins B and L are up-regulated during cancer development for many cancer types and are secreted from tumor cells into the tumor microenvironment (51). A number of studies have correlated increased tumor cathepsin levels with poorer prognosis for cancer patient outcomes (20, 52). Cathepsins involved with tumor cell invasion are, in part, regulated by cystatins. Overexpression of stefin A cDNA in esophageal carcinoma cells inhibited not only *in vitro* invasion but also *in vitro* and *in vivo* growth (53). Overexpression of stefin A decreased *in vitro* invasion by 80 % for these highly invasive carcinoma cells and decreased lung metastasis. A large decrease in intracellular cathepsin B was also seen in this study which showed cathepsin B plays a prominent role in metastasis and tumor growth in this model. The effect of recombinant cystatin C on Caco-2 colon carcinoma cells has also been examined (54). Interestingly, cystatin C had effects on both invasion and growth of the carcinoma cells *in vitro,* and some evidence was presented that cathepsin L may be responsible for these effects.

Cystatin C overexpression has been shown to inhibit cancer cell invasion as well as metastasis (55-57). Konduri *et al.* showed cystatin C overexpression markedly decreases invasion of glioblastoma cells *in vitro* and tumor growth *in vivo* (41). Since tumor growth was also inhibited in this highly invasive type of cancer, it will be important to discover how tumor growth was blocked. Overexpression of cystatin C, and other cystatins, is not directly cytotoxic to cancer cells. Ervin *et al.* did observe higher apoptosis *in vivo* for metastatic melanoma cells which overexpress cystatin C (58). The mechanism for the increased apoptosis has not been determined. Whether an anti-angiogenic effect occurred or some other non-cathepsin cystatin effect is involved remains to be determined. Lung metastasis of human fibrosarcoma cells is dramatically blocked (~90%) in mice by cystatin C overexpression (59). Nude mice were infected with an adenoviral vector expressing cystatin C, where liver was the predominant tissue source of viral cystatin C production. Exogenous cystatin production apparently inhibits both tumor cell extravasation and tumor growth in lung tissues. This experiment demonstrates systemic delivery of a cystatin is effective in metastatic blockade. Tumor cell metastasis to the liver was only slightly decreased; indicating differences in metastatic behavior exists between organs. The reason, however, for this tissue metastatic difference has not been determined.

An issue for cystatin C as an anti-cancer agent is whether it is acting primarily extracellularly or intracellularly as an anti-invasive agent. Since cystatin C is secreted, extracellular action would seem to be more likely as a result of access to cancer cell secreted cysteine proteases. The issue is complicated by evidence indicating a requirement of intracellular cathepsin activity for tumor cell invasion (60, 61). Extracellular matrix proteins, including collagen, are endocytosed and degraded intracellularly in a cathepsin-dependent fashion. As a result, inhibition of solely tumor cell surface associated cysteine proteases by cystatins might be expected to achieve only partial inhibition of tumor cell invasion. Perhaps tumor cell studies with cells derived from cystatin or cathepsin null animals will shed more light on this problem. Multiple species of both cathepsins and cystatins make this a challenging problem for investigation.

Several non-cathepsin mediated actions of the cystatins potentially related to anti-metastatic action have also been uncovered. Cystatin C has been shown to be a TGFβ receptor antagonist acting in a cathepsin inhibitor-independent fashion (19). Cystatin C also inhibits gene expression and cell invasion stimulated by TGFβ in HT1080 cells. This is due to both cathepsin-dependent inhibition of invasion and cathepsin-independent blocking of TGFβ pathway signaling. Phosphorylation of Smad 2, a key TGFβ pathway signaling molecule, could be blocked by cystatin C treatment of fibrosarcoma cells (62). Cystatin C protein was found to bind through its carboxyl region directly to the TGFβ receptor and interfere with activation of receptor signaling, although more work needs to be done to reveal a specific mechanism of action. In addition, a handful of reports have also examined cystatin regulation of cytokine expression in normal cells. In fibroblasts and splenocytes interleukin-6 (IL-6) expression was increased by type II cystatins through an unknown mechanism (63). The cysteine protease inhibitory activity of cystatin was not necessary for the induction of IL-6. Since tumor microenvironments contain host cells, cystatin effects need to be explored in these contexts also. Thus, a new role for cystatins as regulators of cytokine action could have far reaching consequences in cancer due to the pervasive role multiple cytokines play in this disease.

Tumor cell migration is also a critical aspect of invasion. The cysteine proteases appear to be involved in cell migration of certain tumor types but have been found to be less important for others (57) (64). Antisense constructs of cathepsins B or L display decreased tumor cell migrations for osteosarcoma, glioblastoma, and melanoma, to name a few (65-68). The mechanism for cathepsin involvement in cell migration is still unclear, however. Downregulation of the actin binding protein cofilin - or decreased cathepsin B mediated cell detachment have been proposed as possible mechanisms in cathepsin B antisense studies (69). Recent work has also shown that procathepsin X co-localizes with integrin beta 3 on the membrane surface (70, 71). In this way, extracellular cathepsin may help modulate attachment of migrating cells to the extracellular matrix. Because of the many potential cathepsin B targets in cell motility, further genetic and proteomic analysis will provide pivotal data for mechanistic insight on this issue.

Cystatins have been found to inhibit tumor cell migration for a number of different tumor cell types (55, 72). The mechanism for inhibition of tumor cell migration by cystatin is not yet clear, however. Overexpression of cystatin C in tumor cells has not been found to alter cellular adhesion, an important aspect of cell migration (55). Downregulation of cathepsin B or L by various methods has also resulted in decreased migration in certain cancer

cell lines (65, 67, 68). It has not been established that the cystatins inhibit cell motility through cathepsin inhibition. A critical question is whether a cystatin, devoid of cathepsin inhibitor activity, would also be able to inhibit tumor cell migration. This could help determine cathepsin independent action of cystatin on cell migration, perhaps through inhibition of specific cellular signaling pathways. Another possibility is cystatin inhibition of cell motility through calpain (73). Calpain inhibition by cystatins is generally not demonstrated *in vitro*, however, specific conditions may exist in the cell that permit this inhibition (74). Since calpains are intimately involved in cell migration, particularly cell detachment at the rear of the cell, it will be important to investigate this avenue (75). More likely, however, cystatin inhibition of cell migration may be through interference with cell signaling pathways and direct cathepsin inhibition.

Cystatin C null mice which display normal development paradoxically show reduced lung colonization by B16F10 melanoma cells following tail vein injection (76). Subcutaneous growth of melanoma cells in Cystatin C null mice was, however, comparable to control mice. Metastatic inefficiency was attributed to reduced tumor cell seeding of lung tissues as well as inhibition of metastatic tumor cell growth. Perhaps deficient tissue cystatin in these mice allowed melanoma cell mediated cathepsin degradation of critical growth factors required during metastasis, particularly during initial cell seeding. Also, tumor cell adhesion could also be reduced due to less restrained tumor cysteine proteases leading to increased tumor cell death (77). This animal model may provide further insights into specific proteolytic events required during metastasis.

The involvement of cystatins in tumor immunology has not really been investigated. On one hand, the immune system can contribute cysteine proteases to the tumor microenvironment that foster invasion (78). On the other hand, T-cell antigen processing requires cysteine protease activity (particularly cathepsins L and S) that may be inhibited by cystatins (79) (80). A shorter survival time for lung cancer patients with low cathepsin S levels shows a protective role may exist for cathepsin S (80). Additional immune effects of cystatins on interleukin production need to be defined in the context of tumor microenvironments. Highly selective cysteine protease inhibitors may be necessary to attack tumor invasion and growth without stunting natural immune system anti-tumor activity.

5. CYSTATINS AND ANGIOGENESIS

Due to oxygen and nutrient-diffusion limitations for solid tumors, angiogenesis is required for tumor growth beyond 1-2 millimeters (81). Tumor angiogenesis not only provides increased nutrients to permit tumor growth but also supplies tumors with new avenues for metastatic dispersion. Currently, numerous anti-angiogenic stategies are being pursued, with a few at stage III clinical trials (82) (83). Both natural angiogenic responses (wound healing) and those induced by tumors depend upon a change in the local tissue balance of multiple inducers and inhibitors (84). Multiple proteases produced by tumors help overcome the barrier normally afforded by tissue inhibitors that prevent angiogenesis (85). The complex regulation of angiogenesis that exists between proteases and their inhibitors is still being worked out.

The involvement of cystatins in tumor angiogenesis is an emerging story. In fact, knowledge of cystatin and cathepsin involvement in angiogenesis has lagged behind that of other proteases and their inhibitors. Until proof of the involvement of cysteine proteases in angiogenesis could be made, a good case for the participation of cystatins was deficient. Correlations between elevated cathepsin B and tumor vasculature for certain cancer types have been documented in the literature (86). Tumor vasculature-associated cathepsin B could facilitate endothelial cell invasion into tumor stroma and participate in the release of angiogenic factors from extracellular matrices (87). Stimulated migration of endothelial cells by IL8 was shown to be dependent on externalized cathepsin B activity (88). Degradation of matrix-associated angiogenesis inhibitors (TIMPS 1 and 2) by extracellular cysteine proteases is yet another potential mechanism of increased angiogenesis (89). Downregulation of cathepsin B by antisense cDNA expression disrupts angiogenesis induced by glioblastoma cells (90). Gene expression analysis shows both VEGF (vascular endothelial growth factor) and MMP-9 (matrix metalloproteinase 9) to be critically linked to cathepsin B expression. Recent genetic evidence has also shown involvement of cathepsins B, L, and S in tumor angiogenesis. Cathepsins B, L, or S promoted tumor growth in a murine model of pancreatic tumorigenesis (77). The tumor microvascular density declined by about half in pancreatic tumors in CB or CS null mice. Significant increases in apoptosis were also noted in Cathepsin B, L, or S null pancreatic tumors, perhaps, linked to deficient angiogenesis. A portion of tumor-associated cathepsins was shown to be derived from infiltrating immune cells, showing host induction of tumor angiogenesis.

Further support for cathepsin roles in angiogenesis came from work with cathepsin L deficient mice (91). In an ischemic hindlimb model, cathepsin L-deficient mice had reduced neovascularization. Cathepsin L deficient endothelial cells were also shown to be less able to support the vascularization of glioma xenografts. In other studies, cathepsin S-deficient mice produced endothelial cells with reduced invasive capacity through collagen matrices (92). Further work has shown cathepsin S-deficient mice have decreased angiogenesis (93). Cathepsin S was demonstrated to increase the level of certain proangiogenic peptides derived from extracellular matrices. In the same study cystatin C-deficient mice display increased angiogenesis. Interestingly, cystatin C null mice had increased bFGF (basic fibroblast growth factor, FGF2) and IGF-1 (insulin-like growth factor 1) serum levels. Angiogenesis is also stimulated by hypoxia, which has been shown to downregulate cystatins C and stefin B as well as upregulate cathepsin B levels (94). Together cathepsins B, L, and S may be targets of particular interest to thwart tumor angiogenesis and

vascularization. Strong cathepsin involvement in tumor angiogenesis also suggests the cystatins could be potential regulators of therapeutic interest.

Endothelial cells degrade extracellular matrices by both extracellular and intracellular cysteine proteases. Intracellular cathepsins were shown to play a prominent role in *in vitro* tubulogenesis of HUVEC (human vascular endothelial cells) cells through inhibition of the process with cell permeable cysteine protease inhibitors (i.e. CA-074) (95). Overexpression of stefin A inhibited angiogenesis (as well as invasion and tumor growth) of human esophageal squamous cell carcinoma (53). A question of considerable interest is how an intracellular protease inhibitor in tumor cells could influence angiogenesis. It may be that some stefin is released from tumor cells to inhibit extracellular cathepsins. Perhaps a specific peptide processing event that requires intracellular cysteine proteases is involved. It could also be that increased stefin A inhibits secondary lysosomal turnover events in angiogenesis. In light of the report of intracellular cathepsin B activity required for tumor angiogenesis, it will be of interest to see how well intracellular cysteine proteases can serve as targets for anti-angiogenic tumor therapies.

To test cancer treatment potential, a few studies have used synthetic cysteine protease inhibitors as anti-angiogenic agents in animal tumor models. A broad spectrum cysteine protease inhibitor was shown to inhibit tumor angiogenesis (96). More recent work has shown a complex picture for cathepsin B involvement in angiogenesis, which suggests protease targets must be better defined (97). Nonetheless, synthetic cysteine protease inhibitors alone or in conjunction with metalloproteinase inhibitors may be introduced as potential new anti-angiogenic treatments for cancer. In the future perhaps the cystatins too will be harnessed as anti-angiogenic agents in cancer treatment regimens.

6. CYSTATINS AND CELL DEATH

Apoptosis, one type of cell death, is important to tumor progression as well as the response of cancers to therapeutic agents. The importance of apoptosis to cancer is that while many anti-cancer therapies have been found to induce apoptosis, frequently a relative resistance to apoptosis is displayed by transformed cells (98). In general, cancer cells become relatively resistant to apoptosis through mutation and selection of cell variants better able to survive, invade, and proliferate in foreign tissue environments. As seen in Bcl-2 family member overexpression or downregulation of activators of apoptosis, mutations or other genetic events that promote apoptosis resistance may be acquired during tumor progression (99). Changes in the expression of apoptosis mediators favor a shift towards cell survival and are linked to the activation of various oncogenes or loss of tumor suppressors (100-102). The relative resistance to cell detachment triggered apoptosis, termed anoikis, during tumor progression is often another important step towards metastasis (103).

The cystatins have only recently been shown to be involved in normal cell apoptosis, and most dramatically, in selective tissue types. One instance in which cystatins have been linked to apoptosis is in inherited myoclonus epilepsy disease (EPM1) (104, 105). In this disease, a mutation in stefin B results in increased apoptosis of cerebellar granule cells. Cathepsin B appears to mediate the cerebellar apoptosis because cathepsin B null mice have reduced cell losses (106). Besides apoptosis, cathepsins participate in other cell death pathways. As for cancer cells, any cysteine protease dependent cell death pathway could in principal be modulated by cystatins. Cysteine proteases are critical to TNFα induced cell death in immortalized cell lines (107). In fibrosarcoma cells, for example, potential regulation of cell death by cystatins occurs in response to TNFα (108). In this case, lysosomal cathepsin B has been shown to cleave the Bcl-2 family member Bid. Evidence for the cleavage of Bid by cathepsin B to create a pro-apoptotic signal of mitochondrial cytochrome c release has been presented (109). Alternative cell death pathways also exist, depending upon the system. In stefin B deficient mice, which were also deficient in Bid, neuronal apoptosis was not rescued indicating a different pathway was involved (110). A caspase independent cell death pathway has also been recently described for PC12 cells (111). Under conditions of serum starvation, PC12 caspase-independent cell death was mediated through cathepsin D which in turn could be suppressed by cathepsin B. Non-transformed neuronal cells also possess this cell death pathway. As of yet, potential regulation of this pathway has not been explored in regards to the cystatins.

In 2003 Levicar *et al.* bridged a gap between cathepsin L expression and apoptosis in glioblastoma cells (25). Transfection of glioblastoma cells with cathepsin L cDNA inhibited apoptosis through an increase in Bcl-2 expression. Although not yet confirmed, this observation may relate to the nuclear regulation of transcription shown for cathepsin L as described by the work of Goulet *et al.* (112). Determination of the potential role of cystatins in this process will require alteration of endogenous cystatin levels while monitoring cathepsin L effects on gene expression.

The explosive growth of new knowledge on non-apoptotic death pathways has helped uncover information on lysosomal cysteine protease involvement in cell death. Autophagic cell death involves lysosomes, and hence cysteine proteases, however, the potential role of cystatins in this process has not been examined (113). Oxidative damage to cells and other types of cell damage have been shown to rupture lysosomal membranes resulting in the release of cathepsins in non-apoptotic cell death (114-116). Since cathepsins have a rather broad substrate specificity, uncontrolled release of these enzymes from lysosomes would lead to necrotic cell death. Intracellular cystatins could normally inhibit low level lysosomal enzyme leakage, but at higher levels, cystatin inhibitory capacity would be overwhelmed. High cystatin levels in cells would, therefore, be expected to be protective for general cathepsin-mediated cell death. Recently, Petty *et al.*

showed an elevated cystatin C/cathepsin B ratio was associated with chemoresistance in NSCLC patients (117). A question then becomes "Why does decreased cystatin expression occur so frequently in cancers if it could act as a potential anti-death factor ?". Perhaps downregulation of pro-apoptotic factors is a preferred anti-death cancer cell strategy over potential blockade of cathepsin mediated cell death.

Other cancer cell death pathways involving cathepsins which could relate to cystatins have recently been described. Host immune response to tumors is mediated in part by TRAIL (tumor necrosis factor-related apoptosis inducing ligand) - induced apoptosis, and metastatic cells often have increased resistance to TRAIL-induced apoptosis (118). Inhibition of TRAIL-induced apoptosis with cathepsin B specific inhibitor showed the importance of cathepsin B in this cell death pathway (119). Resistance to TRAIL-mediated apoptosis in metastatic oral cancer cells is also suggested by elevated levels of several cystatins (120). Yet another potential cancer cell death pathway is through complement system activation as part of an immune response to tumors. Frade *et al.* showed procathepsin L producing melanoma cells were able cleave C3 complement protein thus producing resistance to tumor cell lysis by complement (121). Extracellular cystatins might interfere with this cancer protective mechanism, but this possibility has not been explored. Another recent report describes a cathepsin dependent, non-apoptotic cell death in apoptosis-resistant glioma tumors by an oncolytic virus (parvovirus H-1) (122). Curiously, viral infected glioma cells increased cytosolic cathepsins while down-regulating cytosolic cystatins. Cathepsin B was activated and relocalized in H-1 infected glioma tumors *in vivo*. The therapeutic benefits of oncolytic viruses would be substantial if novel cell death pathways are employed that could short-circuit apoptosis resistance. The understanding of cystatin involvement with alternative cell death pathways is necessary for future therapies of cancer.

7. FUTURE QUESTIONS AND SUMMARY

Future research questions that relate to the cystatins and cancer include the following: 1. Do the roles of the cystatins change during cancer progression? 2. How do cystatins interact with extracellular matrix proteins? This information could be relevant to proteolytic remodeling of tumor microenvironments. 3. What accounts for differential expression of the cystatins between tumor types? 4. Are certain cystatins 'tumor suppressors' of tumor invasion and, perhaps, even tumor development? Genetic tools are available to pinpoint cancer cell patterns of cystatin gene silencing during cancer cell progression (see accompanying article by Rivenbank and Coleman). 5. What roles do cystatins play in modulating various forms of tumor cell death? 6. What insights can cystatins give us for therapeutic treatments for cancer? Although it may be impractical to consider administration of cystatins to cancer patients directly, cystatins will be a useful tool for gaining insights into blocking cancer invasion-metastasis, and perhaps tumor growth, with synthetic inhibitors targeting various cysteine proteases.

Because cystatins are the major inhibitors of tumor-associated cathepsins, interest in the role of cystatins in cancer will continue to grow. The past few years have brought multiple advances in our knowledge of cystatins' involvement in many aspects of tumor pathobiology. Of chief importance will be a mechanistic understanding of how cystatins serve to promote or inhibit tumor progression and metastasis. New insights into cystatins' role as a cytokine regulator, immunomodulator, and even a regulator of tumor cell gene expression add complexity to diverse aspects of cystatin biology. Undoubtedly, a more complete picture of the role of cystatins in cancer will give impetus towards new approaches to cancer therapeutics.

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