Cysteine Proteinase Inhibitors Stefin A, Stefin B, and Cystatin C in Sera from Patients with Colorectal Cancer: Relation to Prognosis

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ABSTRACT

The levels of cysteine proteinase inhibitors stefin A, stefin B, and cystatin C were determined using ELISAs in sera obtained preoperatively from 345 patients with colorectal cancer and in control sera from 125 healthy blood donors. The levels of stefin A and cystatin C were found to be moderately increased in patient sera (1.4-fold and 1.6-fold, respectively; \( P < 0.0001 \)), whereas the level of stefin B remained statistically unchanged when compared with controls. The medians were 4.3 ng/ml \textit{versus} 3.2 ng/ml for stefin A, 1.2 ng/ml \textit{versus} 1.7 ng/ml for stefin B, and 679 ng/ml \textit{versus} 425 ng/ml for cystatin C. In patient sera, a weak correlation of cystatin C with age \( (r = 0.34; P < 0.001) \) and gender \( (P = 0.01) \) was found. Stefin A and cystatin C levels were independent of Dukes’ stage, whereas stefin B correlated significantly with Dukes’ stage, its level being the highest in stage D \( (P < 0.007) \). Stefin B and cystatin C correlated with survival, whereas stefin A was not a significant prognostic factor in this study. Using medians as cutoff values, patients with high levels of stefin B and patients with high levels of cystatin C exhibited a significantly higher risk of death than those with low levels of inhibitors (hazard ratio = 1.6; 95% confidence interval, 1.2–2.2; \( P = 0.002 \) for stefin B; hazard ratio = 1.3; 95% confidence interval, 1.0–1.8; \( P = 0.04 \) for cystatin C). Our results reveal a correlation between high levels of extracellular cysteine proteinase inhibitors and short survival in patients with colorectal cancer, and the data thus support previous studies suggesting a contributing role of protease inhibitors in the progression of cancer.

INTRODUCTION

CPs have been shown to participate in the dissolution and remodeling of connective tissue and basement membranes in the processes of tumor growth, invasion, and metastasis (1, 2). Increased levels of these enzymes in tumors and in some extracellular fluids are associated with the disease-free and overall survival periods and may therefore serve as prognostic factors for cancer patients (3, 4).

CPs are regulated by endogenous CPIs named cystatins (5, 6). The cystatin superfamily comprises at least four families of closely related proteins, such as steffins (family I), cystatins (family II), kininogens (family III), and various structurally related but noninhibitory proteins of family IV (7, 8). Stefin A and cystatins (family II) share similar structural features, such as molecular size of 11 kDa and 13 kDa, respectively, similar folding of a single polypeptide chain, and lack of carbohydrate moieties. A significant structural difference between steffins and cystatins is the signal peptide, which is responsible for extracellular targeting of cystatins, whereas steffins lack this peptide and have been reported as intracellular inhibitors (9). Stefin A was found in high concentrations in epithelial cells, polymorphonuclear leukocytes, and lymphoid tissue, whereas stefin B is evenly distributed among different cells and tissues (10). Cystatin C is abundant in various tissues and bodily fluids, the highest levels having been determined in cerebrospinal fluid, seminal plasma, and synovial fluid (11). Of all the known CPIs, cystatin C is the most potent inhibitor of CPs, with apparent inhibition constants in the picomolar range (12). The mechanism of inhibition of CPs is well defined (9, 13) from the elucidated X-ray and nuclear magnetic resonance three-dimensional structures of chicken cystatin (14, 15), stefin A (16, 17), and stefin B/papain complex (18).

It has been suggested that steffins and cystatins play a role in several diseases, including cancer, associated with alterations of the proteolytic system (for review, see Ref. 19). Stefin A was the first inhibitor of CP reported to be associated with malignant tumors (10, 20–22). For example, in breast cancer patients, an inverse correlation of stefin A expression with metastatic potential (23) and relapse-free period (24) was suggested. In lung (25) and head and neck tumors (26), stefin A and stefin B levels were moderately increased when compared to the control matched pair tissues, although their higher levels correlated with more favorable patient outcome (25, 27). However, in a recent clinical setting (28, 29), the data are controversial.

The importance of steffins for the cancer patients would be dependent on the expression and function of specific CPs that are regulated by steffins. For instance, the levels of cathepsins B, H, and L have been shown to correlate with disease progression and survival in several tumors (30, 31). Cathepsins are a group of enzymes known to digest extracellular matrix and basement membrane components of the extracellular matrix (for review, see Ref. 32). Cathepsin B is the major cysteine proteinase present in human tumor tissue (33) and is related to the invasion and metastasis of human breast cancer cell lines (34). The elevated levels of cathepsin B have also been identified in adenocarcinoma cells in organ culture (35) and in breast cancer cell lines (36). Cathepsin B is also expressed in mesenchymal sequences of various human tumors, including colorectal cancer (37).

The expression of cathepsin B and its correlation with clinical parameters and prognosis in colorectal cancer patients suggest that cathepsin B is an important marker of tumor growth, invasion, and metastasis. This correlation is consistent with the findings in other tumor types, including breast cancer (30, 31) and lung cancer (38).

The cathepsin B expression level in colorectal cancer tissues is significantly higher than in normal tissues, indicating that cathepsin B may be involved in colorectal cancer progression. The correlation between cathepsin B expression and clinical parameters, such as tumor stage, metastasis, and survival, suggests that cathepsin B may be a prognostic marker for colorectal cancer patients.

The high expression of cathepsin B in colorectal cancer tissues and its correlation with clinical parameters suggest that cathepsin B may be involved in colorectal cancer progression and may represent a potential target for therapeutic intervention. However, further studies are needed to confirm these findings and to elucidate the molecular mechanisms underlying the role of cathepsin B in colorectal cancer.
immunohistochemical analysis of breast tumors (28), the risk of dying was significantly higher in patients with positive staining for stefin A.

Extracellular levels of stefins and cystatin C have also been shown to be associated with cancer progression. The levels of stefin A, increased in sera of patients with hepato cellular carcinoma, correlated with tumor size and number of neoplastic lesions (29). In patients with melanoma, increased cystatin C serum levels correlated with the stage of disease; as a result, they were the highest for metastatic melanoma patients (30). However, the association of extracellular inhibitors with length of survival has not thus far been reported.

Recently, we reported that increased Cat B levels, which were determined in the preoperative sera of 325 colorectal carcinoma patients, correlated significantly with shorter survival (31). In the present study, we measured the levels of stefins A and B and cystatin C in the preoperative sera of the same patient population. Our aim was to investigate the possible correlation of these inhibitors with parameters indicating the progression of the disease to evaluate the biological and clinical relevance of these inhibitors in colorectal carcinoma.

MATERIALS AND METHODS

Patients. Three hundred forty-five patients with histologically verified colorectal cancer were included in the study. On all of them, clinical data, such as age, gender, Dukes’ stage, and survival after the operation, were registered (31). The median Cat B for this patient population was 10.6 ng/ml (range, 1.0–140; Ref. 31). The patient characteristics are shown in Table 1.

The control group for stefin B and cystatin C consisted of 95 healthy blood donors. Their median age was 40 years (range, 19–59). Forty-one were females and 54 were males. For stefin A, the control group of 30 blood donors was used as reported (30). Age and gender distribution were very similar between both control groups.

Sample Collection. Five ml of blood samples were collected preoperatively from patients scheduled to undergo elective colorectal cancer surgery. The blood was clotted at 4°C–8°C and centrifuged subsequently at 3000 rpm. The sera were stored at −80°C until analyzed. Serum from blood donors was sampled in a similar way.

Antigens. Recombinant human stefin A, stefin B, and cystatin C (32, 33) were expressed in Escherichia coli as described and used for the immunization of animals and the preparation of the calibration curves.

Determination of Cystatin C, Stefin A, and Stefin B. Both stefins and cystatin C were analyzed using ELISAs (KRKA d.d., Novo mesto, Slovenia). The components were purified and characterized, and the tests were optimized as described (34, 26, 30).

The intra- and interassay precisions, which were evaluated for serum samples, were in the same range as reported previously for tissue cytosols (26, 35), with coefficient of variance values ranging from 5.2 to 9.5. The recovery was tested by the addition of different amounts of antigens to the serum samples with known antigen concentrations and varied from 88 to 115%. The linearity of ELISAs was tested by serial dilution of serum samples to the levels encompassing the range of the assays. The measured values of diluted samples were compared subsequently with the calibration values. For stefin A and stefin B, ELISA dilutions and calibration curves were parallel within the dilution range of 1:2 to 1:8. For cystatin C ELISA, the same was true for dilutions between 1:20 and 1:150.

A microplate reader (SLT Rainbow, Salzburg, Austria) was used to measure absorbency in ELISAs. The levels of inhibitors were expressed in ng/ml of serum.

Cystatin C ELISA. For the determination of the cystatin C antigen, rabbit polyclonal antibodies (IgG), which were purified from antisera by immunoaffinity chromatography on immobilized cystatin C, were used as capture antibodies (30). Sera at a 1:80 dilution were added to wells of a microtiter plate precoated with rabbit anti-cystatin C IgG. After 2 h of incubation at 37°C, the wells were washed, and murine monoclonal 1A2 anticystatin C antibody, which was purified by affinity chromatography on Protein A-Sepharose and conjugated subsequently with HRP, was added. After a further 2-h incubation at 37°C, 3,3,5,5-tetramethyl benzidine (Sigma, St. Louis, MO) in the presence of hydrogen peroxide was added. The amount of degraded substrate, as a measure of bound immunocomplexed cystatin C, was visualized by absorbance at 450 nm, and the cystatin C concentration was calculated from the calibration curve. The detection limit of the assay was 0.6 ng/ml.

Stefin A ELISA. For stefin A ELISA, murine C5/2 MAb was used for capture, and murine A2/2 MAb, conjugated with HRP, was used for detection (30, 32). Sera in a 1:2 dilution were used in the assay. The procedure was the same as described above for cystatin C. The detection limit of the assay was 0.8 ng/ml.

Stefin B ELISA. For stefin B ELISA, murine A6/2 MAb was used for capture, and murine E7/1 MAb, conjugated with HRP, was used for detection (34, 26). Sera in a 1:2 dilution were used in the assay. The detection limit of the assay was 0.6 ng/ml.

Polyclonal and MAbs, used in assays, recognized equally well native and recombinant antigens. Polyclonal and monoclonal 1A2 antibodies to cystatin C, C5/2 MAb to stefin A, and E7/1 MAb to stefin B bound both complexed and free inhibitors, whereas A2/2 MAb to stefin A and A6/2 MAb to stefin B preferentially bound free inhibitors (26, 34).

Statistical Methods. For descriptive statistics, SPSS PC software was used (Release 6.0, SPSS Inc., Chicago, IL). Differences in the levels of stefins and cystatin C between two or
more groups were tested using rank sum tests. Correlations among the inhibitors’ levels were defined by Spearman rank correlation analysis.

For analysis of survival, the SAS software (Release 6.12, SAS Institute Inc. Cary, NC) was used. Survival curves were estimated using the product limit method of Kaplan-Meier (36), and equality between strata was tested using the log-rank test. Median values were used for the dichotomization of variables into high and low groups. The proportional hazards model of Cox (37) was used for multivariate analysis. Proportionality assumption was verified graphically. In all tests, two-sided Ps < 0.05 were considered significant.

RESULTS

Distribution of Stefin A, Stefin B, and Cystatin C in Patient and Control Sera. The median level of stefin A in patients’ sera was 4.3 ng/ml (range, 0.8–125.4), of stefin B was 1.2 ng/ml (range, 0.6–59.4), and of cystatin C was 679 ng/ml (range, 406–2487). The median levels of inhibitors in control sera were 3.2 ng/ml (range, 1.2–6.4) for stefin A (30), 1.7 ng/ml (range, 0.6–58) for stefin B, and 425 ng/ml (range, 245–686) for cystatin C. Cystatin C and stefin A were significantly higher in the total patient population when compared to control levels (1.4-fold, \( P < 0.001 \), respectively), whereas stefin B was not statistically different. The relative concentrations of stefins and cystatin C in the different Dukes’ stages are shown in Table 2.

Relations between Inhibitor Levels and Other Biological and Clinical Parameters. Correlations between inhibitors and Cat B are shown in Table 3. Cat B is seen to correlate more strongly with stefin B than with stefin A (\( P < 0.001 \); Spearman rank correlation coefficients, \( r = 0.58 \) and \( r = 0.27 \), respectively), whereas only a weak correlation was found between Cat B and cystatin C (\( r = 0.15; P = 0.01 \)).

Stefin B, when treated as a continuous variable, correlates significantly with Dukes’ stage; it is the lowest in stage A and the highest in stage D (\( P < 0.007 \), Kruskall Wallis test). Stefins A and cystatin C levels were independent of Dukes’ stage. Cystatin C levels correlated weakly with the patients’ age, with a rank correlation coefficient \( r = 0.34 \) (\( P < 0.001 \)), and with gender (\( P = 0.01 \), Wilcoxon rank sum test), with a higher level in men.

For the control population, no significant correlation between inhibitor levels and age has been found. The rank correlation coefficient \( r \) was 0.07 for stefin B and 0.04 for cystatin C. Additionally, no significant difference has been found between the inhibitor levels in males and females (\( P = 0.26 \) and \( P = 0.73 \) for stefin B and cystatin C, respectively).

Survival Analysis. The median values were used to dichotomize the levels of stefin A, stefin B, and cystatin C to study their prognostic relevance by univariate and multivariate analysis. For each of these three parameters, overall survival was compared for patients with levels below and equal to or above the median values. Using this cutoff value, stefin B significantly predicted survival (HR = 1.2; 95% CI, 1.0–1.6; \( P = 0.002 \)). As seen from Kaplan-Meier curves (Fig. 1), the probability of survival of patients with lower levels of stefin B is better than for patients with higher levels. Cystatin C levels also correlated significantly with survival in this patient population, although its prognostic impact was lower than for stefin B (HR = 1.3; 95% CI, 1.0–1.8; \( P = 0.04 \)). Patients with cystatin C levels above the median experienced lower survival probability rate than patients with lower levels of this inhibitor (Fig. 1/II). A similar but statistically not significant trend was observed for stefin A (HR = 1.2; 95% CI, 0.9–1.6; \( P = 0.15 \); Fig. 1/II).

The prognostic value of the CPI was compared to those of Dukes’ stage, gender, age, and Cat B by a multivariate analysis. Variables were eliminated from the model singly in a backward fashion with exclusion if \( P > 0.05 \). As seen from Table 4, besides Dukes’ stage, only gender and age were retained in the final model.

We considered whether the combination of variables would increase the prognostic stratification of the patients. Using medians as cutoff values for Cat B (31) and stefin B, we found that the risk was further stratified within the group of patients with

| Table 2 | Distribution of serum stefin A, stefin B, and cystatin C among Dukes’ stages (ng/ml) |
|---------|--------------------------|--------------------------|--------------------------|
| Dukes’ stage | Stefin A | Stefin B | Cystatin C |
| A       | 4.1 | 1.0 | 125.4 | 0.8 | 0.6 | 44.0 | 682.1 | 408.8 | 1544.0 |
| B       | 4.2 | 0.8 | 125.4 | 1.1 | 0.6 | 59.4 | 651.5 | 405.5 | 2486.9 |
| C       | 4.3 | 0.8 | 20.0 | 1.1 | 0.6 | 31.6 | 680.5 | 414.1 | 2373.2 |
| D       | 4.3 | 0.8 | 53.8 | 1.7 | 0.6 | 44.0 | 684.0 | 433.0 | 1937.7 |

\( \text{min.}^a \), minimum; \( \text{max.} \), maximum.

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<tr>
<th>Table 3</th>
<th>Spearman correlation coefficients between selected covariates</th>
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<tr>
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<td>Stefin A</td>
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<td>0.148(^b)</td>
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\( ^a \) Correlation coefficient. 
\( ^b \) \( P \). 
\( ^c \) Number of patients.
high Cat B ($P < 0.005$; Fig. 2I), whereas for patients with low Cat B, the stratification of risk was not significant ($P = 0.36$; Fig. 2II). Patients with high levels of stefin B and Cat B experienced significantly lower survival probability (HR = 2.0) as compared with other combinations or with prognostic values of individual variables. The same trend does not hold for either stefin A or cystatin C.

**DISCUSSION**

Altered regulation of CPs, Cats B, H, and L, is a very distinctive pattern of the malignant cell phenotype (2). Changes in various steps of the production of these enzymes in tumor cells result in increased mRNA and protein expression, extracellular secretion, and alterations in intracellular distribution. The increased levels of enzyme activity, not being balanced by a corresponding increase of CPIs, were suggested as a cause of the remodeling and degradation of extracellular matrix proteins surrounding tumor tissue (2, 4). Enhanced expression and inhibitory activity of CPIs would be expected to diminish the tumor-associated proteolytic activity of CPs, and indeed there is evidence for a suppressive role of the inhibitors of CPs, the stefins, and cystatin C (23–25, 27). Thus, the present results are not consistent with this general view of CPIs because we found the association of higher levels of CPIs with tumor progression and poor prognosis in colorectal cancer patients.

Several studies have compared the levels of CPIs between malignant and normal tissues. Decreased immunoreactivity of stefin A was found in follicular lymphomas (20) and in malignant human tumors of squamous epithelial cell origin (10). Furthermore, during the progression of murine skin papillomas to carcinomas, stefin A mRNA levels decreased (21). Lower mRNA levels of stefin A were found also in some breast tumors (23); however, at the protein level, we recently reported a moderate, 1.4-fold increase of stefin A in cytosols of breast tumors compared with the adjacent control tissue from the same breast (24). In agreement with the latter result, the protein levels of stefin A and stefin B were significantly higher in lung tumors than in control lung tissue (2.0- and 1.3-fold, respectively; Ref. 25). In head and neck tumors, the levels of stefin A and stefin B were not significantly different from those in control adjacent tissue (26), but they were significantly higher in locally advanced than in early tumors (27). However, in breast (24), lung (25), and head and neck (27) tumors, higher levels of stefin A or stefin B were shown to correlate with a favorable prognosis.

The immunohistochemical analysis of stefin A in 384 breast tumors, which was performed by Kuopio et al. (28), was...
the first study to reveal an inverse relation between stefin A and survival in that the risk of death was significantly higher in patients with positive staining of stefin A. Despite the rate of positive cases being relatively low (52 of 440), stefin A was a significant prognosticator also for lymph node-negative patients and, furthermore, the coexpression of stefin A and p53 was a very strong indicator of high risk.

The results on the regulation of CPs by their inhibitors in tumors, as well as clinical relevance of CPIs for prognosis, appear to be contradictory. However, the differences can be explained by the distinct expression of CPIs in neoplastic and nonneoplastic cells and by the different methodologies used for their quantification. On the other hand, the detection of extracellular levels of the inhibitors could serve as an additional approach to clarify the regulation of CPIs during malignancy. Cystatin C is the most abundant and effective extracellular inhibitor of CPs. It was found to be secreted in significant amounts from lung (38) and colon carcinoma cell lines (39). Stefin A can also be secreted into body fluids as shown for patients with hepatocellular (29) and ovarian carcinoma (40). However, in blood, the inhibitory potential of stefins is much lower from that of cystatin C. At least the complexes between stefins and Cat B should be unstable in the concentration range determined in serum ($K_d > 10^{-8}$ M). We found that the levels of stefin A were not significantly changed in the sera of melanoma patients, whereas cystatin C was higher when compared to control sera (30).

The present study reveals the concentration profiles of stefins A and B and cystatin C in the sera of patients with colorectal cancer. In relation to the profiles in tumor tissues, we are aware that the serum levels of CPIs may reflect not only the changes in their local expression in tumors and in protein secretion but also the systemic response to malignant disease. However, as found similarly in tumors, the levels of CPIs were only moderately increased (stefin A, cystatin C) or remained unchanged in malignant sera compared with healthy controls (stefin B). The highest increase was found for cystatin C, and this is consistent with the results found in melanoma (30) and lung cancer. The control group in our study was not age matched with the patients' group, but we did not find any correlation between the levels of inhibitors and age or gender. However, within the group of patients, a weak association of cystatin C with age was found, possibly due to a reduced glomerular filtration rate in older people (41). Because the median age was higher within cancer patients than within controls, this could contribute in part to the increased cystatin C level in patients’ sera. Stefin A and cystatin C were uniformly increased at all stages of the disease, whereas stefin B significantly correlated with the Dukes’ stage, indicating its different regulation and probably different function during malignant progression.

Relating the levels of serum stefins A and B and cystatin C with patient survival, we found, as did Kuopio et al. (28) in breast tumor sections, the association of high levels of the inhibitors with lower overall survival probability. The same trend was observed for all three inhibitors, although for stefin A, the difference between high and low risk patients was not statistically significant. By univariate analysis, stefin B was found to be the strongest prognosticator of all three inhibitors. In multivariate analysis, stefin B and cystatin C were left out from the model, most probably due to their dependence on Dukes’ stage (stefin B) and age (cystatin C). The combination of stefin B and Cat B values further stratified the risk of death within the group of patients with high Cat B (HR = 2.0), whereas for the patients with low levels of Cat B, the difference was not significant. The latter suggests an interaction between these two variables with respect to their association with the survival rate, which should be further validated on an independent set of samples.

The association of extracellular levels of CPIs with malignant progression and survival indicate their biological involvement in colorectal cancer, but the molecular mechanisms of

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Fig. 2 Prognostic significance of the combination of stefin B and Cat B levels in the sera of patients with colorectal cancer. Median levels of both variables were used as cutoff values. I, high Cat B patients; II, low Cat B patients. Bottom, numbers of events and patients at risk at 0, 12, 24, 36, and 48 months.

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4 J. Kos, unpublished results.
altered regulation remain unknown. The results of our study are consistent with recent findings that reveal a correlation of high plasma levels of inhibitors of metalloproteinases and serine proteinases with tumor progression and poor prognosis (42, 43). For tissue inhibitor of metallo-proteinase-1 and plasminogen activator inhibitor-1, it has been proposed that besides protease inactivation, additional tumorigenic functions are contributing to a worse prognosis. The latter has not been found for CPIs, and it seems that high levels of CPIs merely reflect the body’s inadequate response to high CP activity. However, the changes in stability of CP/CPI complexes could contribute to the prognosis of cancer patients. For stefin A, present in high amounts in human sarcoma, a decrease in inhibitory capability was proposed, resulting in impaired binding of CPs (44). On the other hand, posttranslational modifications and alterations in the cellular localization of proteinases could also modify their susceptibility to inhibition. Iacobuzio-Donahue et al. (45) detected Cat B in colorectal carcinoma as a combination of glycosylated and nonglycosylated forms, whereas in premalignant adenomas and normal mucosa, the nonglycosylated form was predominantly present. Multiple forms of Cat B, identified in human lung carcinoma, were more resistant to inactivation by E-64 than Cat B from normal lung tissue (46). Additionally, it has been shown that Cat B from more metastatic lung cells exhibit significantly different rates of inhibition than the enzyme from less metastatic cells (47). One may speculate that the positive effect of up-regulated tumor-associated CPIs is diminished by a decrease in their binding affinity. Recently we found a higher level of Cat B/cystatin C complex being associated with early Dukes’ stages (A and B)5, whereas the concentration of total Cat B was higher in patients with advanced stage tumors (31). This observation is consistent with the hypothesis of hindered inhibitory capability during cancer progression.

In conclusion, contrary to some previous reports, our results reveal a direct relationship between up-regulated levels of CPIs and tumor progression. This is relevant to new therapeutic strategies to balance the increased tumor-associated CPs. Additionally, the association of high levels of CPIs, particularly stefin B with poor survival, could be of clinical importance for the assessment of risk and selection of therapy on the basis of the proteolytic status of the individual colorectal cancer patient.

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