High Preoperative Plasma Tissue Inhibitor of Metalloproteinase-1 Levels Are Associated with Short Survival of Patients with Colorectal Cancer

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ABSTRACT

The objective of the present study was to measure preoperative plasma tissue inhibitor of metalloproteinase (TIMP)-1 levels in colorectal cancer patients and relate these values to clinical and biochemical patient characteristics. TIMP-1 levels were determined by ELISA in EDTA plasma samples collected preoperatively from 588 colorectal cancer patients. Plasma TIMP-1 levels were distributed with a median value of 141.1 μg/liter (range, 53.7–788.7 μg/liter). Whereas no significant differences were found in the median plasma TIMP-1 levels among patients with Dukes’ stage A, B, and C disease, patients with Dukes’ stage D disease had significantly higher plasma TIMP-1 levels (P < 0.0001); however, high plasma TIMP-1 levels were not restricted to advanced disease. A relatively weak correlation between plasma TIMP-1 level and age was found (r = 0.35; P < 0.0001). There was no significant difference in TIMP-1 levels between males and females (P = 0.97). Univariate analysis demonstrated an increasing risk of mortality with increasing TIMP-1 levels [scored as the loge (TIMP-1)]; hazard ratio = 3.3; 95% confidence interval, 2.6–4.2; P < 0.0001]. Including covariates (Dukes’ stage, primary tumor location, gender, age, plasminogen activator inhibitor type 1, and soluble urokinase plasminogen activator receptor) in a multivariate analysis, TIMP-1 was retained in the final model (hazard ratio = 2.5; 95% confidence interval, 1.7–3.7; P < 0.0001). This study showed a highly significant association between preoperative plasma TIMP-1 levels and survival in colorectal cancer patients, with higher plasma TIMP-1 levels being associated with poor outcome. Independent of clinical parameters including Dukes’ stage, plasma TIMP-1 levels were found to strongly predict prognosis of colorectal cancer patients. Additional studies are needed to validate the clinical usefulness of plasma TIMP-1 measurements.

INTRODUCTION

Evaluation of the potential for a given tumor to be invasive and threaten patient survival by metastatic dissemination is a formidable task and is not yet reliably approachable by assessment of any known tumor markers. This problem, however, can perhaps be better approached through collection of data on those marker molecules specifically necessary for the invasive process. For example, aggressive malignant spread of tumor cells requires the secretion of proteolytic enzymes by either the tumor cells or the nonmalignant cells in the surrounding tumor stroma to cleave components of the extracellular matrix and basement membranes (1). High tumor tissue levels of the serine proteinase uPA3 and its receptor (uPAR) have recently been shown to be correlated with shorter survival in patients with colorectal cancer (2, 3) after a similar correlation had been demonstrated in patients with breast cancer (4–6). Recently, we have also demonstrated the prognostic significance of suPAR measured in preoperative plasma of colorectal cancer patients (7). Surprisingly, high preoperative plasma levels of the main uPA inhibitor, PAI-1, have been shown to be associated with shorter survival of colorectal cancer patients (8), and high tumor tissue levels of PAI-1 have in several studies been demonstrated to have prognostic significance in breast cancer (9–12).

The MMPs are a family of zinc-dependent, neutral pH optima, extracellular endopeptidases that can collectively degrade almost all components of the extracellular matrix (13–15). The activity of these proteinases has been demonstrated in normal tissue development and in several pathological conditions. The activities of the MMPs are regulated in situ by a family of low molecular weight endogenous inhibitors referred to as the TIMPs. This family currently consists of four members, TIMP-1, TIMP-2, TIMP-3, and TIMP-4 (16–20). Recent pub-

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3 The abbreviations used are: uPA, urokinase plasminogen activator; uPAR, uPA receptor; suPAR, soluble uPAR; TIMP, tissue inhibitor of metalloproteinase; MMP, matrix metalloproteinase; PAI-1, plasminogen activator inhibitor type 1; HR, hazard ratio; CI, confidence interval.
lications have reported that TIMP-1, in both free and complexed forms, is found in increased amounts in tumor stroma and basement membranes in colorectal cancer tissue (21–24) and that high expression of TIMP-1 in these tissues is correlated to poor prognosis (25). This finding is antithetical to the expected anti-invasive activity of this inhibitor (26, 27) and suggests that TIMP-1 may have additional and as yet poorly characterized biological activities, such as growth stimulation (17, 28–30) and inhibition of apoptosis (31, 32), which were recently demonstrated to be independent of the antiproteolytic activity of the inhibitor. Thus, by stimulating tumor cell growth and/or by inhibiting apoptosis leading to increased survival of malignant cells, high TIMP-1 levels in cancer patients may result in a short survival. It is of interest to note that a similar effect of TIMP-2 in apoptosis could not be demonstrated (32).

In the present study, we measured TIMP-1 levels in preoperatively collected plasma samples from 588 colorectal cancer patients and related these values to patient outcome. This study is the first to demonstrate a highly significant association between preoperative plasma TIMP-1 levels and cancer patient survival independent of clinical parameters including Dukes’ stage.

PATIENTS AND METHODS

Patients. A total of 588 patients undergoing elective surgery for colorectal cancer were included in the study. Blood samples were obtained preoperatively with informed consent from all patients in accordance with the Helsinki Declaration, and the study was approved by the Central National Ethical Committee. All patients had histologically verified adenocarcinoma of the colon or rectum. The diagnosis of colorectal cancer and its stage according to Dukes’ classification (33), with an added D group for cases with metastatic or locally but not radically resected disease, was established by analysis of the resected and immediately fixed (10% phosphate-buffered formalin) bowel and biopsies from lymph nodes and distant metastasis when present. Fifty-eight (10%) patients were classified as having Dukes’ stage A disease, 218 (37%) patients were classified as having Dukes’ stage B disease, 175 (30%) patients were classified as having Dukes’ stage C disease, and 137 (23%) patients were classified as having Dukes’ stage D disease. A total of 338 tumors were colon cancers, and 250 tumors were rectal cancers. Clinical data such as age, gender, primary tumor localization, and survival after surgery were registered. The median observation time of the patients was 6.8 years (range, 5.7–7.9 years), and during the observation period, 375 patients (64%) died. Seventeen deaths that occurred within 1 month of surgery from postoperative complications were censored. Recording of survival for all patients surviving 1 month or more was based on death from all causes. The median age was 69 years (range, 33–90 years), and there were 236 females and 352 males represented in the patient material. Patients with Dukes’ stage A, B, or C disease underwent complete resection of their tumors, whereas patients with Dukes’ stage D disease had resection of their primary tumor and distant metastases whenever possible. None of the patients received adjuvant chemo- or radiotherapy. We have reported previously on the association between plasma PAI-1 (8) and plasma suPAR (7) content and survival in this patient population.

Blood Samples. Blood samples (5 ml) were collected preoperatively from all patients on the day of their operation. Peripheral blood was drawn with minimal stasis and collected in EDTA anticoagulant tubes (Becton Dickinson, Mountain View, CA) in accordance with a previously described protocol (34).

TIMP-1 ELISA. TIMP-1 levels were measured in all EDTA plasma samples by a rigorously tested sandwich ELISA described previously (34). In brief, a sheep polyclonal anti-TIMP-1 antibody (35, 36) was used for catching of the antigen in the ELISA wells. A murine monoclonal anti-TIMP-1 IgG1 (MAC-15; Ref. 37) was used for detection of the antigen, and a rabbit antimouse immunoglobulin/alkaline phosphatase conjugate (Dako, Glostrup, Denmark) was used for the kinetic rate assay. Rate measurements were collected automatically over a 1-h incubation period in a Ceres 900 plate reader (Biotek Instruments, Winooski, VT). A four-parameter fitted standard curve calculated using Kineticale II software was used to determine the TIMP-1 concentration of each sample. Because the monoclonal detection antibody MAC-15 binds both free TIMP-1 and TIMP-1 in complex with MMPs (37), the total TIMP-1 content of the measured sample captured by the sheep polyclonal anti-TIMP-1 antibody (35) was determined by the ELISA. The intra-assay coefficient of variation for 16 replicates of a control citrate plasma pool measured on the same plate was 5.3%, and the inter-assay coefficient of variation for 29 successive assays of the plasma pool (on different days) was 6.2%.

Statistical Methods. The SAS software package (version 6.12; SAS Institute, Cary, NC) was used to manage patient data and perform statistical analyses. The TIMP-1, PAI-1, and suPAR measurements were log transformed (natural log) and treated as continuous variables for the univariate and multivariate survival analyses. The endpoints for survival analysis was death from all causes as obtained from the Danish Death Registry. The Kaplan-Meier method was used to estimate survival probabilities, and the log-rank test was used to test for equality of strata. For graphical representation, the patients were stratified into groups based on the TIMP-1 value, such that each stratum yielded an equal number of events (deaths). This gave approximately equal statistical power to each stratum and served to illustrate a progressive effect of increasing or decreasing levels of TIMP-1. The Cox proportional hazards model was applied for univariate analysis of continuous covariates and for multivariate analysis. The assumption of proportional hazards was verified graphically. Rank statistics were used to calculate correlation coefficients and test hypotheses on location. Tests of independence were done using the $\chi^2$ test. The significance level was set to 5%.

RESULTS

TIMP-1 Levels in Patient Plasma. Using kinetic rate ELISA, TIMP-1 levels were determined in all patient plasma samples ($n = 588$). All of the plasma samples had measurable levels of TIMP-1, and the median TIMP-1 value was found to be 141.1 $\mu$g/liter (range, 53.7–788.7 $\mu$g/liter). The measured TIMP-1 plasma values in the different Dukes’ stages are shown in Fig. 1. The median TIMP-1 level for patients with Dukes’ stage A, B, or C disease was 141.1 $\mu$g/liter (range, 53.7–788.7 $\mu$g/liter). The measured TIMP-1 plasma values in the different Dukes’ stages are shown in Fig. 1. The median TIMP-1 level for patients with Dukes’ stage D disease was 141.1 $\mu$g/liter (range, 53.7–788.7 $\mu$g/liter).
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stage A disease was 120.7 μg/liter (mean, 137.6 μg/liter; SD, 64.8 μg/liter; range, 62.6–419.5 μg/liter). The median TIMP-1 level for patients with Dukes’ stage B disease was 136.8 μg/liter (mean, 159.2 μg/liter; SD, 80.4 μg/liter; range, 53.7–549.8 μg/liter). The median TIMP-1 level for patients with Dukes’ stage C disease was 131.6 μg/liter (mean, 144.6 μg/liter; SD, 58.9 μg/liter; range, 58–410.5 μg/liter), and the median TIMP-1 level for patients with Dukes’ stage D disease was 200.5 μg/liter (mean, 243.5 μg/liter; SD, 143.5 μg/liter; range, 81–788.7 μg/liter). TIMP-1 levels in Dukes’ stages A, B, and C were not significantly different at the 5% level, whereas TIMP-1 levels in patients with Dukes’ stage D were significantly higher ($P < 0.0001$). However, as seen in Fig. 1, a high interindividual plasma TIMP-1 variability was observed in each of the Dukes’ stages; therefore, higher TIMP-1 levels were not restricted to advanced disease. A significant but rather weak correlation was found between plasma TIMP-1 level and the age of the cancer patient ($r = 0.35; P < 0.0001$), whereas there was no significant difference in the levels of TIMP-1 between gender ($P = 0.94$). The rank correlation between TIMP-1 and PAI-1 plasma levels was 0.52 ($n = 580; P < 0.0001$) and 0.53 between TIMP-1 and suPAR ($n = 588; P < 0.0001$). A significant difference was found between plasma TIMP-1 level and primary tumor localization ($P < 0.0001$); patients with colon cancer had significantly higher TIMP-1 plasma levels when compared with patients with rectal cancer.

**Plasma TIMP-1 Levels and Patient Survival.** Treated as a continuous variable, plasma TIMP-1 level (log$_e$ TIMP-1) was found to be significantly associated with survival (Cox proportional hazards model, $P < 0.0001$), and the HR was estimated to be 3.3 (95% CI, 2.6–4.2), indicating the increase in hazard for patients differing by 1 unit on the log$_e$ scale. Patients with higher levels of TIMP-1 in plasma had shorter survival. Kaplan-Meier curves (Fig. 2) were calculated by stratifying the patients into four groups according to the TIMP-1 level found in each individual plasma sample, such that each stratum yielded an equal number of deaths (death from all causes). These four strata were: (a) TIMP-1 ≥ 118.7 μg/liter; (b) 118.7 μg/liter < TIMP-1 ≤ 152.1 μg/liter; (c) 152.1 μg/liter < TIMP-1 ≤ 223.4 μg/liter; and (d) TIMP-1 > 223.4 μg/liter. The survival probabilities at 24 and 48 months were 76% (95% CI, 72–84%) and 61% (95% CI, 54–68%) for stratum I and 34% (95% CI, 25–43%) and 27% (95% CI, 18–35%) for stratum IV. Thus, increasing TIMP-1 level was associated with a continuously increasing risk of mortality.

Dividing patients according to primary tumor localization (colon or rectum) and Dukes’ stage (A, B, C, or D) univariate analysis demonstrated that plasma TIMP-1 levels (log$_e$ TIMP-1) were associated with patient survival in each subgroup. For colon cancer patients, the HR was 2.3 (95% CI, 0.3–32.0; $P = 0.31$) for Dukes’ stage A, 2.9 (95% CI, 1.6–5.5; $P = 0.0007$) for Dukes’ stage B, 2.8 (95% CI, 1.4–5.6; $P = 0.003$) for Dukes’ stage C, and 2.8 (95% CI, 1.7–4.7; $P < 0.0001$) for Dukes’ stage D. For rectal cancer patients, the HR was 1.6 (95% CI,
The patient material was grouped according to primary tumor localization, i.e., rectal cancer and colon cancer. There were 250 rectal cancer patients and 338 colon cancer patients. For both groups, patients were divided into three strata according to the TIMP-1 level (log TIMP-1) found in each individual plasma sample, such that each stratum yielded an equal number of deaths (death from all causes). Figs. 5 and 6 illustrate patient survival for the three patient strata in rectal cancer patients and colon cancer patients, respectively. The levels of plasma TIMP-1 yielding these strata were: (a) TIMP-1 ≤ 143.2 μg/liter, 143.2 μg/liter < TIMP-1 ≤ 193.4 μg/liter, and TIMP-1 > 193.4 μg/liter for Dukes' stage B colon cancer patients; and (b) TIMP-1 ≤ 133.0 μg/liter, 133.0 μg/liter < TIMP-1 ≤ 173.2 μg/liter, and TIMP-1 > 173.2 μg/liter for Dukes’ stage C colon cancer patients.

The patient material was grouped according to primary tumor localization, i.e., rectal cancer and colon cancer. There were 250 rectal cancer patients and 338 colon cancer patients. For both groups, patients were divided into three strata according to the TIMP-1 level (log TIMP-1) found in each individual plasma sample, such that each stratum yielded an equal number of deaths (death from all causes). Figs. 5 and 6 illustrate patient survival for the three patient strata in rectal cancer patients and colon cancer patients, respectively. The levels of plasma TIMP-1 yielding these strata were: (a) TIMP-1 ≤ 115.8 μg/liter, 115.8 μg/liter < TIMP-1 ≤ 151.7 μg/liter, and TIMP-1 > 151.7 μg/liter for rectal cancer patients; and (b) TIMP-1 ≤ 149.9 μg/liter, 149.9 μg/liter < TIMP-1 ≤ 225.3 μg/liter, and TIMP-1 > 225.3 μg/liter for colon cancer patients. For both rectal cancer patients and colon cancer patients, the log-rank test revealed a statistically significant difference (P < 0.0001) in survival in the three patient strata, with a continuously increasing risk of mortality with increasing TIMP-1 level. For univariate analysis of the individual covariates, see Table 1.

Multivariate Analysis. Multivariate Cox analysis of the survival data was performed including Dukes’ stage, primary tumor localization, gender, age, PAI-1 (log PAI-1), suPAR (log suPAR), and TIMP-1 levels (log TIMP-1). In this multivariate analysis, 580 patients with 354 deaths were eligible. The results are shown in Table 1. Consistent with earlier studies, Dukes’ stage was significantly associated with survival; Dukes’ stage D patients had a HR of 7.9 (95% CI, 5.9–10.7) compared to Dukes’ stage B patients, and patients with Dukes’ stage C disease had a HR of 2.3 (95% CI, 1.8–3.1) compared to patients with Dukes’ stage B disease. Patients with rectal cancer were shown to have a shorter survival than patients with colon cancer in the multivariate model (HR = 1.4; 95% CI, 1.1–1.8; P = 0.002). Age scored in years at entry was not significantly correlated with survival (P = 0.06), and neither was gender.
Higher plasma suPAR was correlated with shorter survival \((HR = 1.4; 95\% CI, 1.0–1.9; P = 0.08)\), whereas higher plasma PAI-1 levels were now associated with longer survival \((HR = 0.8; 95\% CI, 0.6–0.9; P = 0.005)\). Finally, the model showed that high plasma TIMP-1 levels were independently associated with a shorter overall survival \((HR = 2.5; 95\% CI, 1.7–3.7; P < 0.0001)\). Thus it was demonstrated that plasma TIMP-1 levels strongly predicted the survival of colorectal cancer patients independent of Dukes’ stage.

### DISCUSSION

This study shows that TIMP-1 levels measured by ELISA in preoperatively obtained plasma samples are significantly associated with survival in patients with colorectal cancer independent of clinical parameters including Dukes’ stage; higher preoperative plasma TIMP-1 levels were found in patients with shorter overall survival. In a multivariate analysis, the plasma TIMP-1 level was found to be a statistically significant and independent variable for patient survival.

The measurements of plasma TIMP-1 levels in colorectal cancer patients were performed using a recently described kinetic rate ELISA (34). This assay enabled specific detection of total TIMP-1 in plasma (EDTA, citrate, and heparin). The assay was tested rigorously, and requirements for sensitivity, specificity, stability, and good recovery were fulfilled. Using the TIMP-1 ELISA, we found in our first study (34) that TIMP-1 levels were elevated in plasma from patients with advanced colorectal cancer (Dukes’ stage D disease) and advanced breast cancer when compared with TIMP-1 levels measured in EDTA plasma from healthy individuals (median, 71.2 µg/liter; range, 58.0–91.8 µg/liter; \(n = 100\)). The present study demonstrates a high degree of interpatient variability, with some patients with early-stage colorectal cancer also having elevated plasma TIMP-1 levels.

Previously published data have shown that TIMP-1 mRNA levels in tumor tissue from patients with colorectal cancer (25) or breast cancer (38) or from patients with non-small cell lung cancer (39) were inversely associated with patient survival. Recent studies using ELISA measurements of total TIMP-1 in tumor tissue extracts from patients with breast cancer demonstrated that high protein levels of the inhibitor were also associated with short survival (40). Furthermore, in an ELISA study by Zucker et al. (41), it was found that patients suffering from advanced gastrointestinal cancer and patients with gynecological cancer had significantly increased plasma levels of MMP-9-TIMP-1 complex when compared with healthy donors. It was found in the same study that for patients with advanced gastrointestinal cancer (stage IV), increased plasma ratios of MMP-9:MMP-9-TIMP-1 complex were significantly associated with shorter survival. In a study of prostate cancer (42), ELISA
measurements of plasma MMP-1, MMP-3, and TIMP-1 showed significantly increased levels of MMP-3 and TIMP-1 in plasma from patients with metastatic disease when compared with the plasma levels found in patients with benign prostate hyperplasia, patients with localized prostate cancer, and healthy donors. However, to our knowledge, the present study including preoperatively collected plasma samples from 588 colorectal cancer patients is the first to demonstrate a strong association between total TIMP-1 levels measured by ELISA in preoperatively obtained plasma samples and survival of patients suffering from early-stage as well as advanced cancer.

The tissue-degrading processes that take place during cancer invasion and metastasis are dependent on the proteolytic activity of MMPs (13–15, 43). Several studies have demonstrated that high levels of these enzymes and their naturally occurring inhibitors are found in both tumor tissue (24, 40, 44) and blood (41, 42, 45) from cancer patients. Our findings of high TIMP-1 levels in plasma collected preoperatively from colorectal cancer patients add to the available information on proteinases and cancer. It could be speculated that the concomitant elevation of both proteinases and their inhibitors in a tumor reflects a high level of activation of the entire proteinase system (both enzymes and inhibitors) initiated during tumor growth and spread, followed by a leak of these proteins into the blood. A rise in the levels of proteinases and their inhibitors in cancer tissue is also seen in the uPA system (46). For example, in studies of colorectal cancer tissue, high levels of both uPA and its inhibitor, PAI-1, have been found to be associated with shorter survival (46). Likewise, it has been demonstrated that high levels of PAI-1 in blood from colorectal cancer patients were significantly associated with poor survival (8). Moreover, it has recently been suggested that PAI-1 might be directly involved in promotion of tumor cell invasion through a mechanism involving facilitation of tumor angiogenesis (47) and/or inhibition of apoptosis (48). Similarly, it has been demonstrated that TIMP-1 has growth-promoting activities by a growth factor-like activity (17, 28, 30) and/or by inhibiting apoptosis (31, 32), the latter of which involves a change in the regulation of apoptosis-mediating gene products. However, a study by Solo-

### Table 1  Univariate and multivariate analysis of individual covariates

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* Dukes’ stage B, C, and D individually compared to A.

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new inhibitor of metalloproteinases from chicken: ChIMP-3. A third


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