Original Article

Serum sE-Selectin Levels and Carcinoembryonic Antigen mRNA-Expressing Cells in Peripheral Blood as Prognostic Factors in Colorectal Cancer Patients

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BACKGROUND: This study analyzed the possible prognostic value of presurgical serum soluble (s)E-selectin levels and/or carcinoembryonic antigen (CEA) mRNA positivity in predicting the disease-free survival of colorectal cancer (CRC) patients. **METHODS:** CEA mRNA (obtained from blood-borne cells by reverse transcriptase-polymerase chain reaction [RT-PCR]), tumor necrosis factor-α (TNF-α), and sE-selectin levels were analyzed in blood samples obtained from 78 patients with primary (n = 62) or recurrent (n = 16) CRC, 40 patients with benign colorectal (CR) diseases, and 78 controls. **RESULTS:** CEA mRNA positivity by RT-PCR was significantly associated with advanced stage ($P < .05$). Median baseline sE-selectin levels were higher in patients with CRC (43 ng/mL) compared with controls (36 ng/mL) or patients with benign CR diseases (31 ng/mL, $P < .001$). These were significantly associated with CEA mRNA positivity by RT-PCR ($P < .05$). Multivariate analysis by forward stepping showed that elevated TNF-α ($P = .001$) and CEA mRNA positivity by RT-PCR ($P = .0001$) were independent predictors of elevated baseline sE-selectin levels. Positive presurgical sE-selectin levels were associated with an increased recurrence rate compared with patients with low levels of this molecule ($P < .001$). Positivity for both CEA mRNA and sE-selectin had a negative prognostic impact, with a 5-year recurrence-free survival rate of 51% compared with 95% of patients with negative parameters ($P < .05$). **CONCLUSIONS:** Detection of presurgical serum sE-selectin levels and CEA mRNA-positive blood-borne cells in CRC patients might provide useful prognostic information in terms of recurrence-free survival, either alone or in combination, and may help in the choice of more aggressive treatment and/or more strict follow-up procedures in high-risk patients. ***Cancer* 2010;116:2913–21.** © *2010 American Cancer Society.*

KEYWORDS: colorectal cancer, blood-borne cells, carcinoembryonic antigen, sE-selectin, metastasis.

Several lines of evidence suggest that the organ selectivity of colorectal cancer (CRC) cells for the liver involves the binding of the metastatic cells to endothelial E-selectin.[1,2] Tumor cell entry into the hepatic microvasculature can trigger a rapid, proinflammatory cascade that begins with increased local tumor necrosis factor-α (TNF-α) production by activated Kupffer cells and leads to up-regulated E-selectin expression on the hepatic sinusoidal vessels,[3,4] which serve not only for initial adhesion and rolling, but also for subsequent diapedesis into metastatic sites.[2] The involvement of TNF-α and E-selectin expression in the metastatic cascade has been also proposed by Sturm et al, who suggested that TNF-α facilitates tumor cell adhesion and extravasation by inducing the expression of E-selectin and other adhesion molecules in the liver vasculature of syngeneic mice.[5]

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It is currently recognized that carcinoembryonic antigen (CEA) is capable of activating hepatic macrophages or Kupffer cells via binding to a CEA receptor.[6] Moreover, the demonstration that CEA itself functions as an alternative receptor for E-selectin to mediate CRC cell adhesion has led to the formulation of a hypothesis concerning the apparent enhanced metastatic potential associated with CEA overexpression on CRC cells and the critical role of selectins in metastatic spread.[7] Accordingly, elevated soluble (s)E-selectin and TNF-α levels have been found in sera from CRC patients, and both TNF-α and serum CEA levels have been independently associated with increased sE-selectin levels.[8]

One of the newer areas being explored in the management of cancer is the use of reverse transcriptase-polymerase chain reaction (RT-PCR) to analyze the blood of cancer patients for the detection of mRNA expressed in tumor cells.[9,10] The CEA gene is 1 of the most widely expressed genes in cancer cells, and serum CEA protein is currently used to follow the course of therapy in the management of colorectal carcinoma.[11] Nonetheless, it has been shown that only approximately half of CRCs shed CEA in levels sufficient for their detection in monitoring therapy.[12] Numerous studies have reported the use of RT-PCR technology to detect CEA message in tumor cells from peripheral blood of cancer patients,[13] suggesting that longitudinal analyses of blood-borne cells expressing CEA by RT-PCR may be useful for the clinical management of CRC and other CEA-expressing tumors in terms of prognosis or in the analysis of the response to therapy either alone[14-17] or in combination with other molecular markers.[18,19]

In light of these considerations, a follow-up study of CRC patients was performed to investigate the possible association of presurgical serum sE-selectin levels with the positivity for CEA mRNA by RT-PCR in peripheral blood cells, as well as their prognostic value in predicting recurrence-free survival.

MATERIALS AND METHODS

Patients

Seventy-eight consecutive patients with CRC (40 men, 38 women; mean age, 58 ± 12 years), treated at the Tor Vergata Clinical Center, entered the study. Patients were histologically diagnosed with primary (n = 62) or relapsing (metastasis to the liver, n = 8; peritoneum, n = 4; lung, n = 2; and multiple metastasis, n = 2) colorectal adenocarcinoma. Primary CRC was pathologically staged according to the International Union Against Cancer TNM Classification: stage I (n = 6), stage II (n = 35), stage III (n = 19), and stage IV (n = 2, with a single resectable liver metastasis). All patients were observed from the time of diagnosis of primary tumor for at least 3 years after surgery or until disease recurrence.

As control groups, 78 healthy age- and sex-matched subjects (mean age, 57 ± 13 years) and 40 subjects with benign colorectal diseases (21 men, 19 women; mean age, 53 ± 13 years) including polyps (n = 28) and chronic ulcerative colitis in stable phase of disease (n = 12) were also evaluated. The study was performed with the appropriate institutional ethics approval and in accordance with the principles embodied in the Declaration of Helsinki, and informed consent was obtained from each subject.

Sample Collection and Processing

Blood samples from primary CRC patients were drawn within 1 week before surgery, or before neoadjuvant chemotherapy and/or irradiation. Samples from patients with metastatic disease were obtained at the time of clinical diagnosis, and before any treatment. Samples from patients with benign disease were drawn at the time of endoscopy. Postsurgical blood samples were obtained from 33 primary CRC patients (stage I, n = 2; stage II, n = 18; stage III, n = 11; and stage IV, n = 2) who provided informed consent to withdraw additional blood samples during follow-up.

After an overnight fast and a rest of at least 20 minutes, blood was drawn from each consenting subject at the middle of vein puncture (to avoid contamination with epidermal epithelial cells) into 7-mL Vacutainers containing no additive or ethylenediaminetetraacetic acid (EDTA). Serum samples were aliquoted, coded, and stored at −40°C until the assays were performed.

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA blood, after dilution in phosphate-buffered saline (PBS), by density gradient centrifugation using Lympholyte-H (CELBIO S.p.A., Siziano, Italy) at 1500 g for 35 minutes. The interface cells were removed, washed in sterile PBS 1×, pelleted, and resuspended in 2 mL of PBS. The cells were then counted and pelleted again at 2500 g for 15 minutes. The cell pellets were dissolved in a denaturing solution containing guanidinium thiocyanate 4 M, Na citrate 25 mM, Sarcosyl NL 30 0.5%, and β-mercaptoethanol 0.1 M and stored at −80°C until RNA extraction.

RNA Extraction and RT-PCR

Total RNA was extracted from PBMCs using QIAampRNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. All RNA preparation and handling steps took place in a laminar flow hood, under RNAse-free conditions. The isolated RNA was resuspended in RNAse-free water and stored at −80°C until assay. The concentration, purity, and amount of total RNA were determined using a DU 530 Life Science UV/Vis Spectrophotometer (Beckman Coulter, Fullerton, Calif). RNA prepared from CEA-producing tumor cell line LS174T was used as positive control. Total RNA was preincubated for 15 minutes at 65°C with DNase (deoxyribonuclease I, amplification grade, Invitrogen Corporation, Carlsbad, Calif) and RNase inhibitor (RNaseOUT, Invitrogen Corporation). After chilling on ice, reverse-transcription of RNA was carried out with the SuperScript III First-Strand Synthesis SuperMix (Invitrogen Corporation), and cDNA was synthesized with 1 to 5 μg of total RNA, 50 μM of oligodT, and Moloney murine leukemia virus reverse transcriptase, according to the manufacturer's instructions. To obtain specific amplification of reverse-transcribed mRNA, cDNAs were amplified using primer pairs for CEA directed to sequences located in 2 different exons. RNA integrity and the fidelity of cDNA synthesis were verified by PCR amplification of the glyceraldehyde phosphate dehydrogenase (GAPDH) housekeeping gene.

CEA and GAPDH primers were designed according to published Ensembl sequence (CEA Ensembl Gene ID ENSG00000105388; GAPDH Ensembl Gene ID ENSG00000111640), using the Universal ProbeLibrary Assay Design Software (https://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp).

These primers were as follows: CEA forward primer, 5′-ACCACAGTCACGACGATCAC-3′; CEA reverse primer, 5′-GGAGTTGTTGCTGGTGATGA-3′; GAPDH forward primer, 5′-TCCACTGGCGTCTTCACC-3′; and GAPDH reverse primer, 5′-GGCAGAGATGAT GACCCTTTT-3′.

PCR was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, Calif). The reaction mixture had a total final volume of 50 μL and contained: 50 ng of cDNA, 0.5 μM of each oligonucleotide primer, a variable amount of distilled water, and 25 μL of HotStarTaq Master Mix (HotStarTaq Master Mix Kit, QIAGEN). PCR was completed after 35 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 60°C, and 1 minute extension at 72°C. An initial denaturation step of 15 minutes at 95°C and a final extension of 10 minutes at 72°C were used.

CEA and GAPDH PCR products, respectively of 210 and 114 bp, were resolved for each patient's sample and cell lines by electrophoresis on 1% agarose in Tris-acetate-EDTA buffer and observed by ethidium bromide staining of the gel. Signals in each CEA transcript were normalized by the corresponding GAPDH transcript and categorized as having negative, moderate (+/++), or strong (+++) intensity using a Bio-Rad molecular imager system (Quantity One 4.6 software, Hercules, Calif).

Immunoassays

Serum CEA and CA 19-9 determination was performed using 2-step chemiluminescent microparticle immunoassays on an ARCHITECT i2000 System (Abbott Labs, Chicago, Ill). Serum CA 72-4 levels were measured using a 2-step chemiluminescent microparticle immunoassay on an Elecsys 2010 System (Roche Diagnostics GmBH, Mannheim, Germany). The cutoff limits chosen for sample evaluation were 5 ng/mL, 37 U/mL, and 6 U/mL for CEA, CA 19-9, and CA 72-4, respectively. Serum TNF-α and sE-selectin levels were measured by enzyme-immunometric assays (R&D Systems, Minneapolis, Minn) according to the manufacturers' instructions. Cutoff limits were calculated on the basis of the 95th percentile of the values observed in healthy subjects and were set at 20 pg/mL and 70 ng/mL, respectively. Measurements were done blinded. All samples were assayed in duplicate, and those showing values above the standard curve were retested with appropriate dilutions.

Statistical Analysis

Data are generally presented as percentage of positive patients. Unless otherwise specified, antigen levels are expressed as median and interquartile range (IQR). Statistical analysis was performed by Kruskal-Wallis test followed by Mann-Whitney *U* test for post hoc comparison. Differences between percentages were assessed by cross-tabulation tables and chi-square analysis. Univariate and multivariate regression analyses were performed to assess the possible associations between variables. Recurrence-free survival was calculated by the Kaplan-Meier method, and the significance level was assessed according to the Cox-Mantel test. The time to the endpoint was calculated from the date of surgery until the event date, with the event being any recurrence, locoregional or systemic, or

the latest day of follow-up. A sample size of 62 patients participated in the follow-up study, with a 90% power to detect a 50% difference in survival rates between patients with either positive or negative variables. Only P values $<.05$ were regarded as statistically significant. All calculations were made using a computer software package (Statistica 8, StatSoft Inc., Tulsa, Okla).

RESULTS

CEA mRNA from blood cells of patients with different CRC stages was analyzed by RT-PCR before surgery, or before neoadjuvant chemotherapy and/or irradiation. Fifty-one (65%) of 78 patients with different stages of colorectal cancer had blood cells that were positive for CEA mRNA by RT-PCR, whereas none of 40 patients with benign colorectal diseases was positive; 2 of 78 apparently healthy individuals were marginally positive (data not shown). A significant overall association was found between tumor stage and RT-PCR analysis for CEA in the blood of CRC patients, with 54% CEA mRNA positivity by RT-PCR in patients with early stages compared with 78% in patients with advanced stages (chi-square, 5.25; $P = .022$). Conversely, no significant association was found between CEA mRNA positivity by RT-PCR and serum CEA levels (Mann Whitney test, $P = .159$). CEA mRNA positivity was found in 37 (63%) of 59 patients with CEA-negative serum, compared with 14 (74%) of 19 patients with CEA-positive serum (chi-square, 0.76; $P = .382$).

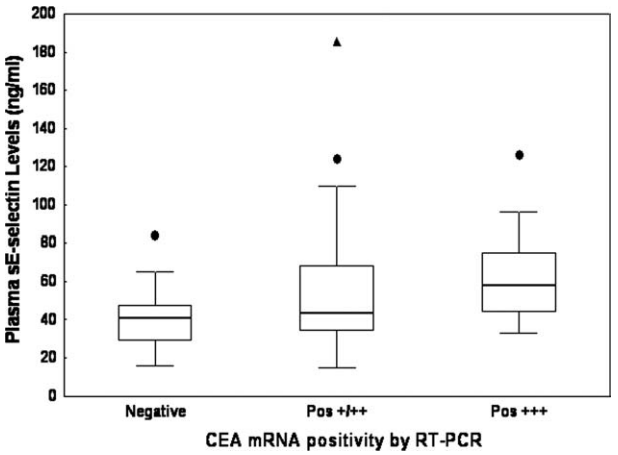

Figure 1. Box plot analysis of sE-selectin levels in colorectal cancer patients stratified on the basis of negative, moderate (+/++), or strong (+++) carcinoembryonic antigen (CEA) mRNA positivity (pos) by reverse transcriptase-polymerase chain reaction (RT-PCR) is shown. Data are presented as median values (solid lines), interquartile range (columns), and nonoutlier (whiskers) ranges. Closed circles indicate outliers; closed triangles, extreme. Kruskal-Wallis analysis of variance and median test: $H = 7.48$; $P < .03$.

Median baseline sE-selectin levels were higher in patients with CRC (median [IQR]: 43 [34-62] ng/mL) compared with controls (36 [25-46] ng/mL) or patients with benign colorectal diseases (31 [25-46] ng/mL, $H = 17.1$, $P = .0002$). Increasing levels of baseline sE-selectin were significantly associated with CEA mRNA positivity by RT-PCR ($H = 7.48$, $P = .024$) (Fig. 1). In particular, only 1 (4%) metastatic patient negative for CEA mRNA before surgery was positive for sE-selectin at baseline (84.2 ng/mL), compared with 29% of CEA mRNA-positive patients (chi-square, 7.16; $P = .007$). To assess the possible determinants of baseline sE-selectin levels among the clinical and laboratory features of CRC, a multivariate regression analysis was performed in which adjustments were made for the following variables: age, sex, site of primary tumor, grade, stage of disease, serum CEA, CA 19-9 and CA 72-4 tumor markers, CEA mRNA, and TNF-α levels. The final model by forward stepping showed that elevated TNF-α (regression coefficient, 0.371; $P =$

Table 1. Multivariate Regression Analysis of Baseline sE-Selectin Levels in Colorectal Cancer Patients

Predictor Variable	Univariate Analysis		Multivariate Analysis	
	β-Coefficient (SE)	*P*	β-Coefficient (SE)	*P*
Sex	0.048 (0.118)	.688	Out	
Age	−0.126 (0.125)	.315	Out	
Stage of disease	−0.002 (0.167)	.992	Out	
Site of primary tumor	−0.142 (0.139)	.314	Out	
Grade	0.052 (0.145)	.723	Out	
CEA mRNA	0.428 (0.125)	.001	0.449 (0.110)	.0001
Serum CEA levels	0.051 (0.151)	.736	Out	
Serum CA 19-9 levels	0.055 (0.158)	.726	Out	
Serum CA 72-4 levels	−0.052 (0.145)	.721	Out	
Serum TNF-α levels	0.322 (0.128)	.015	0.371 (0.110)	.0014

SE indicates standard error; CEA, carcinoembryonic antigen; TNF-α, tumor necrosis factor-α.

.0014) and positivity by RT-PCR (regression coefficient, 0.449; $P = .0001$) were independent predictors of elevated levels of sE-selectin (Table 1).

The interactions among baseline sE-selectin, TNF-α levels, and CEA mRNA positivity by RT-PCR were further analyzed by 2-way analysis of variance. As shown in Figure 2, increased baseline levels of sE-selectin were significantly associated to CEA mRNA positivity in patients with elevated TNF-α levels ($F = 4.68$, $P = .012$). Post hoc analysis by Bonferroni test showed that median presurgical sE-selectin levels did not significantly differ among patients with negative TNF-α levels and either negative or positive CEA mRNA expression in peripheral blood (Fig. 2). Conversely, median presurgical sE-selectin levels of patients with positive TNF-α levels and negative CEA mRNA expression in peripheral blood were significantly lower (46.8 ng/mL) than those observed in patients with moderate (88.9 ng/mL, $P < .001$) or strong (126.5 ng/mL, $P < .001$) CEA mRNA positivity (Fig. 2).

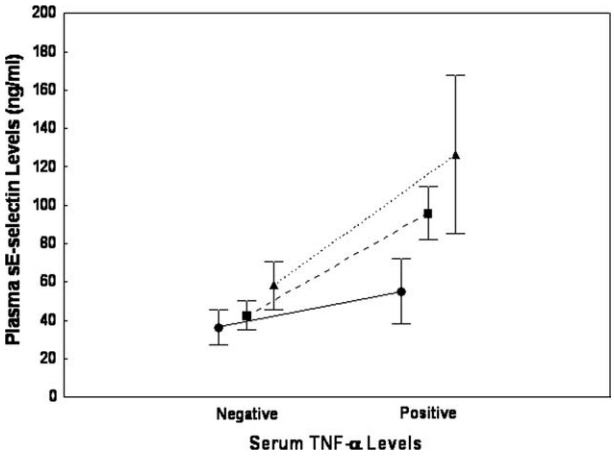

Figure 2. Two-way analysis of variance analysis of sE-selectin levels in colorectal cancer patients stratified on the basis of carcinoembryonic antigen (CEA) mRNA positivity by reverse transcriptase-polymerase chain reaction (RT-PCR) is shown, with positive (>20 pg/mL) or negative (≤20 pg/mL) tumor necrosis factor-α (TNF-α) levels. Closed circles indicate patients negative for CEA mRNA by RT-PCR; closed squares, patients with moderate CEA mRNA positivity by RT-PCR; closed triangles, patients with strong CEA mRNA positivity by RT-PCR; vertical bars, 95% confidence intervals. $F = 4.68$, $P < .02$.

Clinical information on postoperative follow-up was available from all primary CRC patients. Over a median follow-up period of 3 years, 49 (79%) of the 62 patients remained free of disease, whereas 13 (21%) patients experienced relapsing disease (stage II, n = 3; stage III, n = 9; stage IV, n = 1; median follow-up: 20 months, range, 4-103). No differences were observed in age, sex, grade, or site of primary tumor between patients with and without recurrence (data not shown). Presurgical sE-selectin levels were above the cutoff in approximately 46% of the relapsing patients, compared with 6% (chi-square, 13.3; $P = .0003$) of the patients who remained free of disease. Multivariate regression analysis was then performed including recurrence as the dependent variable and age, sex, grade, site of primary tumor, stage of disease, CEA mRNA, and presurgical sE-selectin, TNF-α, CEA, CA 19-9, and CA 72-4 tumor marker levels as the independent variables. The final model by forward stepping showed that both stage of disease (regression coefficient, 0.408; $P = .0005$) and elevated presurgical sE-selectin levels (regression coefficient, 0.432; $P = .0002$) were independent predictors of recurrent disease (Table 2).

sE-Selectin level and CEA mRNA RT-PCR analysis during postsurgical follow-up was performed in 33 primary CRC patients. As shown in Table 3, sE-selectin levels significantly decreased during postsurgical follow-up in patients in whom positive CEA mRNA by RT-PCR

Table 2. Multivariate Regression Analysis of Disease Recurrence Rates in Colorectal Cancer Patients

Predictor Variable	Univariate Analysis		Multivariate Analysis	
	β-Coefficient (SE)	*P*	β-Coefficient (SE)	*P*
Sex	0.091 (0.118)	.445	Out	
Age	0.104 (0.123)	.402	Out	
Stage of disease	0.320 (0.144)	.031	0.408 (0.109)	.0004
Site of primary	0.147 (0.137)	.288	Out	
Grade	0.026 (0.142)	.854	Out	
CEA mRNA	0.161 (0.138)	.249	Out	
Serum CEA levels	0.077 (0.132)	.563	Out	
Serum CA 19-9 levels	0.014 (0.135)	.918	Out	
Serum CA 72-4 levels	−0.076 (0.135)	.575	Out	
Serum TNF-α levels	0.196 (0.133)	.146	Out	
Baseline sE-selectin levels	0.341 (0.142)	.021	0.432 (0.109)	.0002

SE indicates standard error; CEA, carcinoembryonic antigen; TNF-α, tumor necrosis factor-α.

Table 3. Baseline and Postsurgical sE-Selectin and CEA Levels of 33 Patients With Primary CRC Categorized According to the Changes Observed in CEA mRNA Positivity by RT-PCR During Postsurgical Follow-up

CEA mRNA Positivity	Follow-Up, Mean mo	Median sE-Selectin Levels			P^a	Median CEA Levels			P^a
		Baseline, ng/mL (IQR)	Postsurgical, ng/mL (IQR)	% Change		Baseline, ng/mL (IQR)	Postsurgical, ng/mL (IQR)	% Change	
Increased, n = 9; recurrences, n = 1	42	40 (31-44)	37 (28-62)	+13%, from −11% to 21%	.37	2.7 (1.6-4.0)	2.6 (1.5-2.8)	−10%, from −30% to 75%	.91
Unmodified, n = 15; recurrences, n = 4	40	38 (28-54)	45 (23-53)	+13%, from −27 to 33%	.36	3.4 (1.5-4.7)	3.0 (2.1-6.7)	+30%, from −13% to 150%	.21
Decreased, n = 9; recurrences, n = 2	44	49 (40-64)	36 (31-40)	−30%, from −44% to −11%	.04	2.8 (1.8-10.8)	2.6 (1.8-7.2)	+23%, from −38% to 100%	.86
Kruskall-Wallis ANOVA test for % change				$H = 6.2$, $P = .05$				$H = 1.1$, $P = .58$	

CEA indicates carcinoembryonic antigen; CRC, colorectal cancer; RT-PCR, reverse transcriptase-polymerase chain reaction; IQR, interquartile range; ANOVA, analysis of variance.
[a]Wilcoxon test for paired data: baseline versus postsurgical.

Figure 3. Kaplan-Meier analysis of recurrence-free survival time of colorectal cancer patients is stratified on the basis of (A) sE-selectin levels (Cox-Mantel test, 3.5; $P < .001$) or (B) carcinoembryonic antigen (CEA) mRNA positivity by reverse transcriptase-polymerase chain reaction (RT-PCR) (Cox-Mantel test, 2.37; $P < .02$).

became negative. Conversely, those patients in whom negative CEA mRNA became positive, or who had a persistent CEA mRNA positivity by RT-PCR during follow-up, did not show any significant change of sE-selectin. No differences were observed for CEA levels (Table 3).

Figure 3A demonstrates the Kaplan-Meier recurrence-free survival curves for nonmetastatic CRC patients with low (below the cutoff value) or high (above the cutoff value) presurgical sE-selectin levels. As shown, high presurgical sE-selectin levels were associated with an increased recurrence rate compared with patients with low levels of this molecule in the overall CRC population (Cox-Mantel test, 3.5; $P = .0005$). Similar results were obtained when analysis was restricted to a subgroup of stage II only patients (Cox-Mantel test, 2.6; $P = .01$).

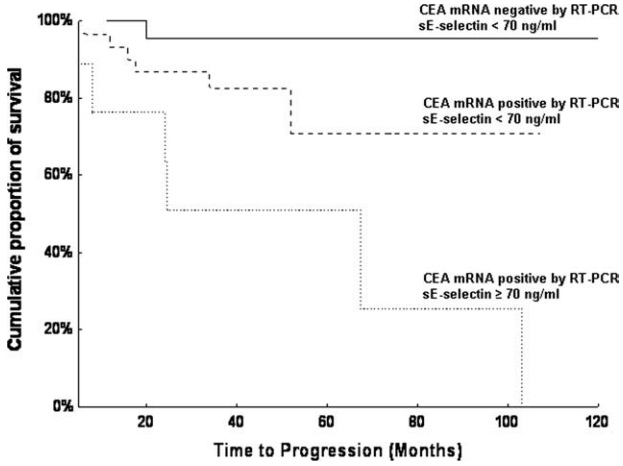

Figure 4. Kaplan-Meier analysis of recurrence-free survival time of colorectal cancer patients is stratified on the basis of positive sE-selectin levels (> the cutoff value of 70 ng/mL) and/or carcinoembryonic antigen (CEA) mRNA positivity by reverse transcriptase-polymerase chain reaction (RT-PCR) (log-rank statistic, 8.71; $P < .02$).

Similarly, CEA mRNA positivity by RT-PCR was associated with an increased recurrence rate compared with negative patients (Cox-Mantel test, 2.37; $P = .018$) (Fig. 3B). CEA mRNA and presurgical sE-selectin were both positive or negative in 9 and 23 patients, respectively, whereas CEA mRNA positivity was associated with negative presurgical sE-selectin levels in the remaining 30 patients. Figure 4 shows recurrence-free survival Kaplan-Meier curves for patients stratified according to CEA mRNA positivity by RT-PCR and presurgical serum sE-selectin levels. As shown, positivity for both CEA mRNA and sE-selectin before surgery had a negative prognostic impact, with a 5-year recurrence-free survival of 51%; patients with positive CEA mRNA but negative presurgical sE-selectin had a 5-year recurrence-free survival rate of 71%, whereas patients with negative CEA mRNA and presurgical sE-selectin had a 5-year recurrence-free survival rate of 95% (chi-square, 8.71; $P = .013$).

DISCUSSION

The ability of tumor cells to secrete macrophage-activating soluble factors or activate Kupffer cells through cell-cell contact has been investigated as to their role in determining the nature of the hepatic host proinflammatory response and thereby in regulating the arrest, localization, and fate of the metastasizing cells.[3,4] In particular, recent experimental studies have demonstrated that CEA is capable of triggering a molecular cascade beginning with increased local production of TNF-α and culminating in up-regulated expression of E-selectin on the endothelium.[6,7]

Here we report for the first time that detection of blood-borne cells expressing CEA mRNA represent a prognostic factor for CRC recurrence, as previously suggested by other authors,[16-18] but mostly in patients with elevated baseline sE-selectin levels. In fact, the 5-year survival rate of primary CRC patients with positive CEA mRNA expressing cells in the peripheral blood, but negative presurgical sE-selectin levels (71%), was significantly higher than that of patients with both positive CEA mRNA and positive sE-selectin (51%, Cox-Mantel test, 2.29; $P = .02$). This observation may partially explain the negative conclusions reached by other authors, who suggested that the presence of circulating tumor cells might be of little value as a prognostic factor, because they appeared not to be associated with conventional prognostic factors, namely the presence of metastatic disease.[20] The findings here of a significant association between increased sE-selectin and positive CEA mRNA-expressing cells in the peripheral blood of patients with low-grade inflammatory status (as evidenced by increased levels of TNF-α) fuel the hypothesis that circulating tumor cells may, indeed, represent a prognostic factor, but only in those patients in whom they are capable of triggering the molecular changes demonstrated in the studies cited above,[6,7] and suggest that circulating tumor cells might be responsible for macrophage activation in vivo, release of proinflammatory cytokines, and up-regulation of vascular E-selectin, which can be detected in its soluble form on shedding from the endothelial cell surface. This hypothesis is also consistent with the finding that the highest sE-selectin levels were detected in patients highly expressing CEA mRNA and with positive TNF-α levels compared with patients with negative levels of both variables.

Of interest, CEA mRNA positivity by RT-PCR, but not CEA protein levels, predicted the occurrence of vascular inflammation, as evidenced by increased sE-selectin levels, in multivariate analysis. This might be because of the occurrence of normal CEA levels in a high percentage of our patients (24%) and its lack of association with the positivity of its mRNA in peripheral blood cells. It must be noted that, overall, we observed a correlation between serum CEA and sE-selectin levels ($P = 0.28$, $P < .05$), as previously reported,[8] but this association was completely lost at multivariate analysis. We are aware that, given the multiple pathophysiological changes known to be associated with cancer, statistical correlation does not

necessarily indicate a causal relationship, and we cannot conclusively define the events predisposing to endothelial activation and E-selectin up-regulation. However, we might hypothesize that blood-borne CEA-expressing tumor cells, or their released products, may play a crucial role in triggering the host proinflammatory response, thus facilitating tumor cell adhesion, rolling, and diapedesis of the inflamed endothelium at metastatic sites.

Although the results reported here do not allow us to provide a definite answer on the causal relationship between blood-borne tumor cells and soluble E-selectin, the present study provides further evidence on the prognostic value of circulating tumor cells for the recurrence-free survival time of CRC patients, as previously suggested by other authors.[16-19] In addition, we demonstrate for the first time that presurgical serum sE-selectin levels may also behave as a useful prognostic indicator for recurrent CRC. This finding is in agreement with previously published observations indicating that elevated sE-selectin concentration was associated with a bad prognosis for recurrence-free survival and overall survival in patients with lymph node-negative breast cancer,[21] and that E-selectin could be detected on the membranes of peritumoral endothelial cells, but not on breast epithelial cells, using PCR in situ hybridization.[22] Finally, the results presented here suggest that a combination of both presurgical blood-borne CEA-expressing tumor cells and sE-selectin levels might offer a better prognostic indication than either marker alone.

These hypotheses require detailed experimental evaluation before their ultimate significance can be determined. Nevertheless, we hope that our study will prompt investigators to design new studies to better understand the relationship between CEA and sE-selectin in disease progression, as well as their contribution as a prognostic factor for CRC. Although our study may be limited by the small sample size, the results reported here strongly suggest that detection of CEA mRNA-positive blood-borne cells in CRC patients with vascular inflammation might provide useful prognostic information in terms of recurrence-free survival, and may help in the choice of more aggressive treatment and/or more strict follow-up procedures in a subgroup of patients who are at high risk of recurrence. New prospective studies specifically designed to address this issue are warranted.

CONFLICT OF INTEREST DISCLOSURES

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