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Association between Serum Carcinoembryonic Antigen and Endothelial Cell Adhesion Molecules in Colorectal Cancer

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Key Words

Adhesion · Colorectal cancer · Interleukin-6 · Metastasis · E-selectin, soluble · Tumour necrosis factor-α · Vascular cell adhesion molecule-1

Abstract

Objectives: To analyse the behaviour of pre-surgical serum levels of soluble (s)E-selectin and vascular cell adhesion molecule (sVCAM) in patients with colorectal cancer, and to evaluate their possible correlation with carcinoembryonic antigen (CEA), pro-inflammatory cytokines and clinicopathological features with respect to their prognostic value in predicting metastatic disease. ***Methods:*** Pre-surgical serum levels of sE-selectin, sVCAM, interleukin-6 (IL-6), IL-1β, tumour necrosis factor-α (TNF-α) and CEA were measured in 194 patients with colorectal adenocarcinoma, 40 patients with benign colorectal diseases and 59 healthy subjects. ***Results:*** sE-selectin, sVCAM, TNF-α and IL-6 levels were significantly higher in patients with colorectal cancer compared to either healthy subjects or patients with benign disease. Positive rates of sE-selectin, sVCAM and TNF-α levels were significantly associated with Dukes' stage D colorectal cancer, and all three variables were independently associated to the presence of distant metastases. Positive sE-selectin, sVCAM and TNF-α levels were significantly associated to CEA. TNF-α and CEA levels were independently related to the presence of positive levels of sE-selectin and/or sVCAM. ***Conclusions:*** Our findings suggest that the host inflammatory response to cancer cells, and/or their released products (i.e. CEA), might be responsible (via cytokine release) for the elevation in circulating adhesion molecules in patients with colorectal cancer.

Introduction

Carcinoembryonic antigen (CEA), the best known and most widely used serum tumour marker, is a glycoprotein that has been experimentally implicated in the development of hepatic metastases of human colorectal cancer [1]. Serum CEA levels are controlled by the production rates of the tumour, its location and stage, size, differenti-

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ation, vascularity, presence or absence of distant metastases, but also by the rate of its elimination [2–4]. It is well known that Kupffer cells in the liver are capable of binding CEA through a specific receptor [5–7], thus clearing soluble CEA from the circulation. CEA binding and internalisation by Kupffer cells are consistent with the mechanism of receptor-mediated endocytosis [8] and results in the release of a series of cytokines [9]. Using an in vitro model, it has been recently demonstrated that the culture of mouse Kupffer cells with CEA resulted in the release of interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α). Moreover, E-selectin was expressed on endothelial cells co-cultured with Kupffer cells and CEA, resulting in increased adhesion of colorectal cancer cells through sialylated Lewis antigens (sLe) [10].

The recognition of E-selectin by carcinoma cells expressing sLe determinants is considered an essential step for their adhesion to activated endothelium, which precedes the haematogenous spread of cancer [11–15]. Stronger adhesion to the endothelium is mediated through other classes of adhesion molecules such as the vascular cell adhesion molecule (VCAM), a cytokine-inducible endothelial cell adhesion molecule of the immunoglobulin supergene family [16]. Increased expression of E-selectin and VCAM has been found in small vessels surrounding lesions in colorectal cancer [17–20], with the highest expression being noted in metastatic sites [20]. Furthermore, elevated levels of circulating isoforms of selectins have been recently described in some malignant diseases [21–28]. Although little investigated so far, soluble forms of selectins have been related to the clinical behaviour of human tumours. In particular, soluble (s) E-selectin and sVCAM levels have been found to be significantly elevated in metastatic compared with non-metastatic colorectal cancer patients [21, 25, 27], whilst different results were reported by Velikowa et al. [26] who showed high serum levels of sVCAM, but not sE-selectin in patients with colorectal carcinoma. Moreover, elevated pre-surgical serum levels of these adhesion molecules were not independent prognostic factors for patient survival [21].

Here we report the results of a longitudinal study on 234 patients with benign or malignant colorectal diseases aimed to analyse the behaviour of pre-surgical serum levels of sE-selectin and sVCAM in patients with different stages of colorectal cancer, and to evaluate their possible correlation with CEA, pro-inflammatory cytokines (IL-6, TNF-α and IL-1β) and clinicopathological features with respect to their prognostic value in predicting metastatic disease.

Patients and Methods

Patients

Two hundred thirty-four consecutive patients with either benign (n = 40) or malignant (n = 194) colorectal diseases treated at our institutions entered into the study. As a control group, 59 healthy donors (35 males, 24 females; mean age 52.2 ± 10.9 years, ranging from 33 to 71) were also evaluated. The 194 colorectal cancer patients (107 males, 87 females; mean age 58.9 ± 9.8 years, ranging from 33 to 71) were histologically diagnosed with primary (n = 178) or relapsing (metastasis to the liver: n = 9, peritoneum: n = 3, lung: n = 2, and multiple metastasis: n = 2) colorectal adenocarcinoma. Primary colorectal cancer was pathologically staged according to the Dukes' classification: stage A (n = 16), stage B (n = 100), stage C (n = 51) and stage D (n = 11, with a single resectable liver metastasis). Benign colorectal diseases (21 males, 19 females; mean age 52.7 ± 13.1 years, ranging from 32 to 70) included adenomas (n = 2), polyps (n = 26) and chronic ulcerative colitis in stable disease (n = 12).

One hundred and sixty-five of the 178 patients diagnosed with primary colorectal cancer (94 males, 71 females, mean age 60.1 ± 8.9 years; stage A = 14, stage B = 95, stage C = 45, and stage D = 11) were followed from the time of diagnosis of primary colorectal cancer for at least 3 years after surgery, or until diagnosis of recurrent disease. All patients were generally reviewed at 3-month intervals during the first 2 years after surgery. Thereafter, the interval between visits increased to 6 or 12 months in parallel with tumour stage. The study was approved by the ethical committees of our institutional boards, and informed consent was obtained from each subject.

Sample Collection and Immunoassays

Serum samples from primary cancer patients were obtained within 1 week before surgery, or prior to neoadjuvant chemotherapy and/or irradiation. Samples from patients with metastatic disease were obtained at the time of clinical diagnosis, and prior to any treatment. Serum samples from patients with benign disease were drawn at the time of endoscopy. All samples were coded and stored at –40 °C until the assays were performed.

Serum CEA levels were measured using a commercially available immunoassay (Abbott Labs, Chicago, Ill., USA). The cut-off limit used for serum sample evaluation was set at 5 ng/ml.

Serum sVCAM and sE-selectin levels were measured by enzyme-immunometric assays (both from R&D Systems, Minneapolis, Minn., USA) according to the manufacturer's instructions. Intra- and inter-assay coefficients of variation for both assays were below 5 and 10%, respectively. The minimum detectable doses were 0.5 ng/ml for sVCAM, and 0.1 ng/ml for sE-selectin.

Serum IL-6, TNF-α and IL-1β levels (all from R & D Systems) were measured by enzyme-immunometric assays according to the manufacturer's instructions. Intra-assay coefficients of variation were below 5% for both IL-6 and TNF-α, and below 8% for IL-1β. Inter-assay coefficients of variation were below 10% for all three assays. The lower detection limits of the assays were 0.7, 4.4 and 1 pg/ml for IL-6, TNF-α and IL-1β, respectively.

Measurements were done blinded. All samples were assayed in duplicate and those showing values above the standard curve were re-tested with appropriate dilutions.

Statistical Analysis

Data are presented as means ± SD, or as medians and interquartile ranges. Statistical analysis was performed by Anova and unpaired

Table 1. Pro-inflammatory cytokine and adhesion molecule levels in patients with benign (n = 40) or malignant (n = 194) colorectal diseases and 59 healthy subjects

		Healthy subjects	p value	Colorectal diseases			
				benign	p value	malignant	p value[a]
sE-selectin, ng/ml	Mean ± SD	37.1 ± 15.7	NS	35.5 ± 16.6	<0.0001	58.7 ± 28.1	<0.0001
	Median (IQR)	34.4 (24.9–47.0)		31.2 (24.6–45.6)		50.6 (37.7–74.7)	
sVCAM, ng/ml	Mean ± SD	379 ± 179	NS	336 ± 140	<0.0005	542 ± 354	<0.001
	Median (IQR)	352 (265–485)		311 (235–386)		455 (327–602)	
IL-6, pg/ml	Mean ± SD	3.0 ± 5.5	<0.05	3.1 ± 4.9	<0.01	8.3 ± 20.8	<0.0001
	Median (IQR)	0.7 (0.1–2.8)		1.6 (0.7–3.3)		3.0 (1.4–6.2)	
TNF-α, pg/ml	Mean ± SD	3.8 ± 9.2	<0.05	8.3 ± 16.9	<0.05	12.6 ± 42.3	<0.0001
	Median (IQR)	0.1 (0.1–2.5)		2.7 (0.1–7.0)		4.4 (2.3–8.0)	
IL-1β, pg/ml	Mean ± SD	0.9 ± 1.7	NS	0.5 ± 0.5	NS	0.6 ± 0.5	NS
	Median (IQR)	0.2 (0.1–1.2)		0.3 (0.2–0.9)		0.5 (0.3–0.8)	

p values indicate the level of significance obtained for post-hoc analysis for multiple comparison performed by Newman-Keuls or Mann-Whitney U tests. IQR = Interquartile ranges.

[a] Malignant colorectal disease vs. healthy subjects.

t test. When necessary, log transformation was used to normalise the data, or appropriate non-parametric tests were employed (Kruskal-Wallis and Mann-Whitney U tests). Multiple regression analysis was used to assess relationships between variables. Differences between percentages were assessed by crosstabulation tables and χ^2 analysis. Univariate and multivariate analyses were performed by Cox's proportional hazard model: the first step was performed by the log rank test, then the covariates found to be associated with distant metastasis were included in the Cox's regression model. The outcome variable referred to the final status 'free of disease'/metastasis as 0 or 1. For each variable, the proportional hazard has been tested. Clinical and laboratory variables considered in the analysis were: age, sex, tumour location, grading, tumour stage, serum levels of CEA, cytokines and plasma adhesion molecule levels. The variables that achieved statistical significance in the univariate analysis were subsequently included in a multivariate analysis using a Cox regression model. Only p values lower than 0.05 were regarded as statistically significant. All calculations were made using computer software packages (Statistica, StatSoft, Tulsa, Okla., USA, and EGRET Cytel Software, Cambridge, Mass., USA).

Results

Table 1 shows the results obtained for all the analytical variables measured in samples obtained from 293 subjects, including 59 normal donors and patients with either malignant (n = 194) or benign (n = 40) colorectal diseases. Plasma sE-selectin (Anova: $F = 29.4$, $p < 0.00001$) and sVCAM (Anova: $F = 11.9$, $p < 0.0001$) levels were significantly higher in patients with colorectal cancer compared to either healthy subjects or patients with benign disease, whereas no difference was observed for both variables between the two latter groups (table 1). Patients with colorectal cancer also had the highest levels of serum TNF-α and IL-6 levels, followed by patients with benign disease and normal donors (Kruskal-Wallis Anova median test: TNF-α: $H = 30.8$, $p < 0.0001$; IL-6: $H = 35.6$, $p < 0.0001$). No significant differences were observed for serum IL-1β levels among the three groups (table 1) and within the three groups with respect to gender and age (data not shown).

Of interest, median sE-selectin, sVCAM and TNF-α levels were significantly elevated in patients with distant metastases compared with Dukes' stage A (TNF-α: $p < 0.01$; sE-selectin: $p < 0.05$; sVCAM: $p < 0.0005$), stage B (TNF-α: $p < 0.002$; sE-selectin: $p < 0.05$; sVCAM: $p < 0.0001$) and stage C (TNF-α: $p < 0.0001$; sE-selectin: $p < 0.05$; sVCAM; $p < 0.0001$) colorectal cancer, whereas only a trend was observed for median IL-6 levels ($p = 0.06$; table 2).

sE-selectin, sVCAM, IL-6 and TNF-α levels were considered positive if above the 95th percentile of the levels observed in the 59 healthy subjects. This resulted in cut-off values of 54 ng/ml, 604 ng/ml, 13 pg/ml and 24 pg/ml for sE-selectin, sVCAM, IL-6 and TNF-α, respectively. As shown in table 3, no significant association was found between positive rates of all the analytical variables measured and tumour location, grading or lymph node involvement, whereas positive rates of sE-selectin ($p < 0.05$), sVCAM ($p < 0.005$) and TNF-α ($p < 0.001$) levels

Table 2. Summary of the evaluation of sE-selectin, sVCAM, IL-6 and TNF-α in colorectal cancer patients

Dukes' stages	Patients	sE-selectin ng/ml mean ± SD	sVCAM ng/ml mean ± SD	IL-6 pg/ml median (IQR)	TNF-α pg/ml median (IQR)
A	16	47.1 ± 17.5	516 ± 227	1.8 (1.1–2.4)	4.5 (3.3–6.1)
B	100	58.4 ± 27.4	486 ± 250	3.2 (1.5–5.7)	4.6 (2.6–7.9)
C	51	56.2 ± 27.1	487 ± 268	2.9 (1.5–6.0)	3.1 (1.3–5.5)
D[a]	27	71.5 ± 34.0	862 ± 629	4.6 (1.4–10.2)	10.1 (4.8–43.4)
p value		<0.05	<0.001	0.06	<0.002

IQR = Interquartile range.
[a] Including 16 relapsing patients.

Table 3. Association of positive rates of sE-selectin, sVCAM, IL-6 and TNF-α with different pathological variables in 194 colorectal cancer patients

Variable	Patients	Positive levels							
		sE-selectin (>54 ng/ml)	p value	sVCAM (>603 ng/ml)	p value	IL-6 (>13 pg/ml)	p value	TNF-α (>24 pg/ml)	p value
Tumour location[a]									
Colon	53	23 (43)		11 (21)		8 (15)		5 (9)	
Rectum	85	35 (41)		18 (21)		6 (7)		3 (4)	
Sigma	40	19 (48)	NS	9 (23)	NS	9 (22)	NS	0 (0)	NS
Grading[a]									
1	21	7 (33)		7 (33)		5 (24)		1 (5)	
2	131	59 (45)		23 (18)		15 (11)		6 (5)	
3	26	11 (42)	NS	8 (31)	NS	3 (12)	NS	1 (4)	NS
Tumour size[a]									
T1–T2	62	21 (34)		17 (27)		3 (5)		3 (5)	
T3–T4	116	56 (48)	NS	21 (18)	NS	20 (17)	<0.02	5 (4)	NS
Lymph node involvement[a]									
N0	116	48 (41)		26 (22)		13 (11)		4 (4)	
N+	62	29 (47)	NS	12 (19)	NS	10 (16)	NS	4 (7)	NS
Tumour stage									
A	16	4 (25)		5 (31)		1 (6)		1 (6)	
B	100	44 (44)		21 (21)		12 (12)		3 (3)	
C	51	22 (43)		9 (18)		9 (18)		2 (4)	
D	27	19 (70)	<0.05	15 (56)	<0.005	6 (22)	NS	10 (37)	<0.001
Distant metastasis									
No	167	70 (42)		35 (21)		22 (13)		6 (4)	
Yes	27	19 (70)	<0.01	15 (56)	<0.001	6 (22)	NS	10 (37)	<0.001
Serum CEA levels									
Negative	138	57 (41)		32 (23)		15 (11)		9 (7)	
Suspicious	18	6 (33)		2 (11)		3 (17)		0 (0)	
Positive	38	26 (68)	<0.01	16 (42)	<0.05	10 (26)	NS	7 (18)	<0.05

Serum CEA levels were considered negative if <5 ng/ml; suspicious if ranging between 5 and 10 ng/ml, and positive if >10 ng/ml.
Numbers in parentheses represent percentages.
[a] 16 patients with relapsing disease were excluded from the analysis.

were significantly associated with the stage of disease. In particular, positive levels of all three molecules were found in patients with metastatic disease compared to those without (table 3). Positive rates of IL-6 were found only in patients with large tumours ($p < 0.02$). Of interest, a significant association was observed between positive serum levels of sE-selectin ($p < 0.01$), sVCAM ($p < 0.05$) or TNF-α ($p < 0.05$) levels and CEA (table 3). Therefore, to further analyse the relationship between cytokines, adhesion molecules and CEA levels in colorectal cancer, a multiple regression analysis including age, sex, tumour stage, TNF-α, IL-6, sE-selectin, sVCAM and serum CEA levels was carried out. The final model obtained by stepwise regression analysis showed that both TNF-α (β-coefficient = 0.22, $p < 0.01$) and CEA (β-coefficient = 0.15, $p < 0.05$) levels were independently related to the presence of positive levels of sE-selectin and/or sVCAM ($R^2 = 0.11$, $p < 0.0001$). Moreover, stage (β-coefficient = 0.64, $p < 0.0001$), TNF-α (β-coefficient = 0.21, $p < 0.0001$), CEA (β-coefficient = 0.15, $p < 0.002$) and elevated levels of sE-selectin and/or sVCAM (β-coefficient = 0.10, $p < 0.05$) were independently associated with the presence of distant metastases ($R^2 = 0.63$, $p < 0.0001$).

Clinical information on post-operative follow-up was available from 165 primary colorectal cancer patients, pathologically staged as: stage A (n = 14), stage B (n = 95), stage C (n = 45), and stage D (n = 11, with a single resectable liver metastasis). Over the follow-up period, 113 (68.5%) of the 165 patients remained free of disease (median follow-up: 64 months), 3 had a second primary tumour while 52 (31.5%) patients experienced recurrence of disease (18 local recurrences and 31 distant metastases). The association between laboratory parameters and disease-free survival of patients who developed distant metastases was assessed by Cox's proportional hazard model using the above-mentioned cut-off values. Univariate analysis of clinicopathologic variables and pre-surgical blood levels of sE-selectin, sVCAM, TNF-α, IL-6, and serum CEA followed by the step-down selection of variables revealed that only stage (hazard ratio = 3.4, 95% confidence interval: 2.2–5.3, $p < 0.001$) and pre-surgical serum CEA levels (hazard ratio = 2.9, 95% confidence interval: 1.4–5.9, $p < 0.005$) had an independent prognostic role in predicting metastatic disease. Moreover, univariate and multivariate analyses of the same variables in the 95 patients with stage B colorectal cancer confirmed the independent prognostic role of pre-surgical serum CEA levels for recurrence (hazard ratio = 1.01, 95% confidence interval: 1.003–1.016, $p < 0.05$).

Conclusions

The results obtained in this study demonstrated that pre-surgical sE-selectin and sVCAM levels were significantly higher in patients with colorectal cancer compared with either healthy controls or patients with benign diseases. Furthermore, analysis of colorectal cancer patients demonstrated that these adhesion molecules were not related to tumour size, grading or lymph node involvement, but were independently associated to the presence of distant metastasis, which accounted for the significant association found between sE-selectin or sVCAM and the Dukes' stage of disease.

To date, the source and biological significance of sE-selectin and sVCAM in colorectal cancer is not yet known. The possibility of a cytokine-induced release of soluble adhesion molecules from endothelial cells has been suggested by Velikova et al. [26] in colorectal cancer patients, based on an evident correlation between serum sE-selectin concentration and the total white blood cell count. It is well known that tumour cells and/or tumour-associated leukocytes and platelets may produce inflammatory cytokines, such as IL-6 and TNF-α [29]. Circulating levels of these cytokines have been associated with the disease status of colorectal cancer patients [30–34], and it has been suggested that IL-6 is an independent negative prognostic marker of survival in this neoplastic disease [31]. In particular, elevated serum levels of IL-6 have been found in patients with large tumours [33] and metastases [31, 33]. Moreover, it has been shown that blood concentrations of IL-6 correlated with the amount of circulating CEA [31]. In the present study, elevated serum IL-6 levels were found in patients with T3–T4 tumours, but were not related to the presence of visceral metastasis or CEA levels. No significant association was found between IL-6 levels and disease-free survival, as previously suggested by other authors [34]. Conversely, TNF-α levels showed positive correlations with the presence of metastatic disease and CEA levels, suggesting that CEA might play a direct role in the mechanism(s) inducing TNF-α elevation in colorectal cancer.

The recent finding that culture of Kupffer cells in the presence of CEA results in the release of TNF-α in the culture medium, and that treatment of endothelial cells with the latter results in increased expression of E-selectin and enhanced adhesion of colorectal cancer cells to the endothelium [10] establishes the potential for a previously unrecognised biologic role for CEA in inflammation and haematogenous tumour spread. In the present study, positive sE-selectin, sVCAM and TNF-α levels were all signif-

icantly correlated to CEA, and both TNF-α and CEA levels were independently related to the presence of positive levels of endothelial adhesion molecules. These results are consistent with the hypothesis that the in vitro model proposed by Minami et al. [10] might be actually working in vivo, and that the host inflammatory response to cancer cells, and/or their released products (i.e. CEA), could be responsible – through cytokines release – for the up-regulation of adhesion molecules on endothelial cells, thus favouring the haematogenous spread of colorectal cancer cells. The finding that distant metastasis, but not lymph node involvement, correlated with elevated sE-selectin and sVCAM levels is also consistent with this hypothesis, and further suggests that soluble adhesion molecules may play a pivotal role in the pathogenesis of blood-borne metastasis.

We are aware that, given the multiple pathophysiological changes known to be associated with cancer, statistical correlation does not necessarily indicate a biological relationship, and we cannot conclusively define the clinical impact of sE-selectin and sVCAM determination on the management of colorectal cancer patients. A prospective study focusing on the longitudinal determination of adhesion molecules in colorectal cancer patients is needed before any conclusion can be drawn. However, we believe that the measurement of soluble adhesion molecules in colorectal cancer patients may add useful information to elucidate the mechanisms involved in the haematogenous spread of colorectal cancer cells and might help in developing better treatment regimens to prevent the development of metastatic disease.

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