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Original Paper

Elevated Serum E-selectin in Patients with Liver Metastases of Colorectal Cancer

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E-selectin, an endothelial cell adhesion molecule, mediates the initial step of leucocyte adhesion to activated vascular endothelium. The soluble isoform of E-selectin promotes angiogenesis in rat cornea. In the present study, we investigated whether leucocyte adhesion and angiogenesis are also involved in tumour progression and metastasis of colorectal cancer. Therefore, we determined the level of circulating soluble E-selectin in serum samples of 38 patients with colorectal cancer; 20 patients with non-metastatic and 18 patients with metastatic disease. Median levels of soluble E-selectin were found to be significantly higher in metastatic tumour disease (88.7 ng/ml, range 25–203 ng/ml) than in healthy controls (34.9 ng/ml, range 15–59 ng/ml, $P = 0.01$), in patients with primary tumours or with local recurrences (39.5 ng/ml, range 22–100 ng/ml). Furthermore, there was no correlation with the serum level of C-reactive protein, fibrinogen or tumour necrosis factor α suggesting that the elevation of E-selectin is independent of inflammation in tumour patients. Therefore, we propose that elevated soluble E-selectin may reflect increased neovascularisation in metastatic tumour tissue. Copyright © 1996 Elsevier Science Ltd

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INTRODUCTION

THE INTERACTION of tumour cells with vascular endothelium is crucial for tumour invasion and metastasis [1, 2]. Initial adhesion of cells to activated vascular endothelium is mediated by the endothelial adhesion molecule E-selectin, a member of the selectin family [3, 4]. Selectins are a group of sialic acid-binding molecules present on activated endothelium (E-selectins), platelets (P-selectins), and leucocytes (L-selectins) [5, 6]. It has been shown that colorectal tumour cells bind to E-selectin *in vitro* [7], indicating that E-selectin-mediated binding of tumour cells to vascular endothelium might be involved in haematogenous metastasis. Recently, sialyl-Lewis X has been identified as an E-selectin ligand [3, 8]. Sialyl-Lewis X represents an oncofetal antigen that is highly expressed in adenocarcinoma [9, 10]. Expression of E-selectin on intratumoral vessels in malignant melanoma as well as sialyl-Lewis X on colorectal cancer tissue has been shown to be associated with reduced survival of tumour patients [11, 12].

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Recently, a circulating isoform of E-selectin has been described which was increased in inflammatory and some malignant diseases, e.g. gastrointestinal cancer and lymphoma [13, 14]. It was also shown to promote angiogenesis in rat cornea [15]. In the present study, we determined the serum levels of soluble E-selectin in patients with primary colorectal tumour lesions, local recurrences and metastatic disease of colorectal cancer in order to determine whether serum levels of E-selectin are associated with metastatic disease in colorectal cancer.

MATERIALS AND METHODS

Serum samples

Freshly frozen sera of colorectal cancer patients treated at the First Department of Internal Medicine, University of Mainz, were tested. Serum samples were taken from 38 patients with colorectal cancer: 20 patients (11 females, 9 males, median age 59 years, range 40–71 years) had primary tumour lesions ($n = 14$) or local recurrences ($n = 6$). 18 patients (10 females, 8 males, median age 61 years, range 48–75 years) had colorectal tumour metastases to the liver, 3 with additional metastases either to the lymph node ($n = 1$) or the bone

($n = 2$). Tumour staging was defined by at least two diagnostic methods including computed tomography, ultrasound or magnetic resonance imaging at the time the blood was taken. The diagnosis was confirmed during clinical follow-up for at least 6 months. Tumour cell morphology was graded as well to moderately differentiated and as poorly or undifferentiated tumour cells. In patients with primary tumours or local recurrences, 8 tumour tissues were well or moderately differentiated and 12 were poorly differentiated. In patients with metastatic disease, 9 tumours were well or moderately differentiated and 9 tumours were poorly or undifferentiated. 20 healthy individuals (median age 31 years, range 23–41 years) were analysed as controls.

Enzyme-linked immunosorbent assay

Soluble E-selectin was measured with a commercially available ELISA assay following the manufacturer's instruction (Bender MedSystems, Vienna, Austria). An E-selectin monoclonal antibody recognising the extracellular region of E-selectin was precoated on to microtitre wells. Standards and diluted samples were introduced into the wells and immediately a horseradish peroxidase-conjugated anti-E-selectin was added. Soluble E-selectin in the patients' serum bound to the coated antibody, while the second antibody bound to another epitope of E-selectin. Following 2 h incubation, unbound enzyme-conjugated anti-E-selectin was removed by washing and substrate solution was added to the wells. A coloured product was formed in proportion to the amount of soluble E-selectin in the sample. The absorbance at 450 nm was measured by an ELISA reader (Multiscan plus, LabSystems, Finland). A standard curve was prepared from the E-selectin standard and test samples were assessed from this standard. Serum C-reactive protein (CRP) was measured by nephelometry (Behring nephelometer analyser, Behringwerke Marburg, Germany). Fibrinogen was determined in a coagulometer (Zeiss, Germany). The normal range of serum CRP was 0–0.5 mg/dl, and of fibrinogen 150–450 mg/dl. Serum tumour necrosis factor (TNF)α was measured by ELISA (genzyme, U.S.A.) and found to be within the normal range of 5–20 pg/ml.

E-selectin-adhesion assay

Binding of tumour cells to E-selectin was analysed in a spot-adhesion assay employing a recombinant E-selectin-immunoglobulin chimera produced in COS cells as previously described [7]. Falcon 1008 dishes were incubated with a spot of 50 µl goat-anti-human IgG (Sigma, Germany) in a concentration of 10 µg/ml in 50 mM Tris, pH 9.5, for 90 min. Dishes were washed three times with PBS and blocked with 1% bovine serum albumin at 4°C overnight. Subsequently, the dishes were incubated with 1 ml of cell culture supernatant, containing 5 µg/ml E-selectin-fusion protein, and incubated for 30 min at room temperature. The promyelocytic cell line HL60, that is known to express the E-selectin ligand to a high degree, was washed with PBS and resuspended at a concentration of 10^6 cells/ml in a solution of 150 mM NaCl and 2 mM $CaCl_2$. The cells were allowed to bind for 30 min and were washed three times with the buffer described above. Adherent cells per cm^2 were independently counted under a phase contrast microscope (Zeiss, Germany) by two different investigators.

Inhibition of E-selectin-binding was tested using the probes of three healthy controls (soluble E-selectin 35, 30, 40 ng/ml) and 3 patients with metastatic colorectal cancer (soluble E-selectin 203, 200, 156 ng/ml). In the inhibition assay, HL60 cells were preincubated with sera samples at a 1:5 dilution for 1 h at 37°C. E-selectin-binding of HL60 in the absence of serum was taken as 100%.

Statistics

The software CSS:STATISTICA (StatSoft) was used to analyse the data. The correlation between the serum E-selectin level and the serum level of CRP, fibrinogen and TNFα was analysed by the non-parametric Mann-Whitney test. Correlation analyses were performed using Kendall's tau. A significance limit of 0.05 was used.

RESULTS

All patients and normal controls had detectable serum levels of soluble E-selectin. The median concentration was highest in patients with liver metastases of colorectal cancer (88.7 ng/ml; range 25–203 ng/ml, standard deviation ±50.3) compared with normal subjects (34.9 ng/ml; range 15–59 ng/ml, standard deviation ±12.8) and non-metastatic colorectal cancer (39.5 ng/ml; range 22–100 ng/ml, standard deviation ±15.8) (Figure 1). There was a significant difference between the median serum level of patients with metastatic colorectal cancer and normal subjects ($P = 0.009$) or patients with non-metastatic colorectal cancer ($P = 0.012$). There was no difference between the median serum level of healthy controls and patients with non-metastatic colorectal cancer ($P = 0.1$). 3 patients with additional bone or lymph node metastases had very high serum levels with 203, 200, 156 ng soluble E-selectin/ml, respectively.

To determine whether a high concentration of circulating E-selectin depends either on tumour cell morphology or is derived from an existing inflammation in the patient's body, 17 moderately differentiated tumours and 21 poorly differentiated or undifferentiated tumours were examined. No correlation between grading and metastasis and the level of circulating E-selectin was observed. In order to determine whether soluble E-selectin occurs in the circulation only in response to an inflammation, three markers of inflammation, namely

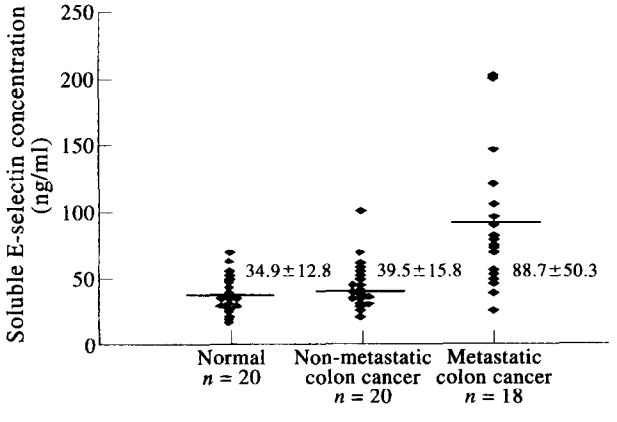

Figure 1. Serum levels of soluble E-selectin (ng/ml) in healthy subjects ($n = 20$) (median 34.9 ng/ml) (range 15–59 ng/ml, standard deviation ±12.8), patients with non-metastatic colorectal cancer ($n = 20$) (39.5 ng/ml) (range 22–100 ng/ml, standard deviation ±15.8) and patients with metastatic colorectal cancer ($n = 18$) (median 88.7 ng/ml) (range 25–203 ng/ml, standard deviation ±50.3). Bars indicate the medians. Each sample was tested four times and the mean value is given.

CRP, fibrinogen and TNFα were determined in patients and healthy controls. Serum CRP was elevated in 19 out of 38 patients, serum fibrinogen was increased in 6 out of 38 patients and TNFα in 3 out of 38 patients. None of the 12 healthy controls showed elevation of any of these inflammatory markers. Although quite a number of patients showed an increase in markers of inflammation, no correlation could be observed between that increase and the presence of either metastasis or an increased level of soluble E-selectin. These results are illustrated in Figure 2, showing the correlation between soluble E-selectin and CRP ($r = 0.1$) or soluble E-selectin and TNFα ($r = 0.0$) in patients with metastatic or non-metastatic colorectal cancer.

It is still unknown whether soluble E-selectin in colorectal cancer comprises the entire extracellular domain of the protein and if this isoform retains binding capacity. To address this question, we examined whether sera with circulating E-selectin are capable of inhibiting the adhesion of HL60 cells to immobilised E-selectin. Three sera with high levels of circulating E-selectin derived from patients with metastatic colon cancer were used. Uninhibited HL60 binding to immobilised E-selectin was taken as 100%. Figure 3 illustrates that sera of tumour patients, diluted 1:5, inhibit HL60 cell

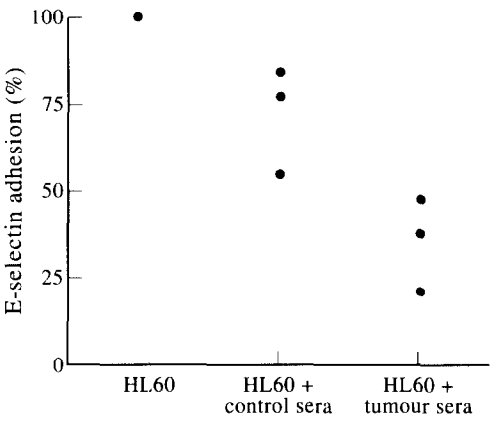

Figure 3. Effect of three samples each of normal and colon cancer sera on E-selectin binding of HL60 cells. Uninhibited HL60 cell binding was taken as 100%. Each sample was tested three times and the mean value is given.

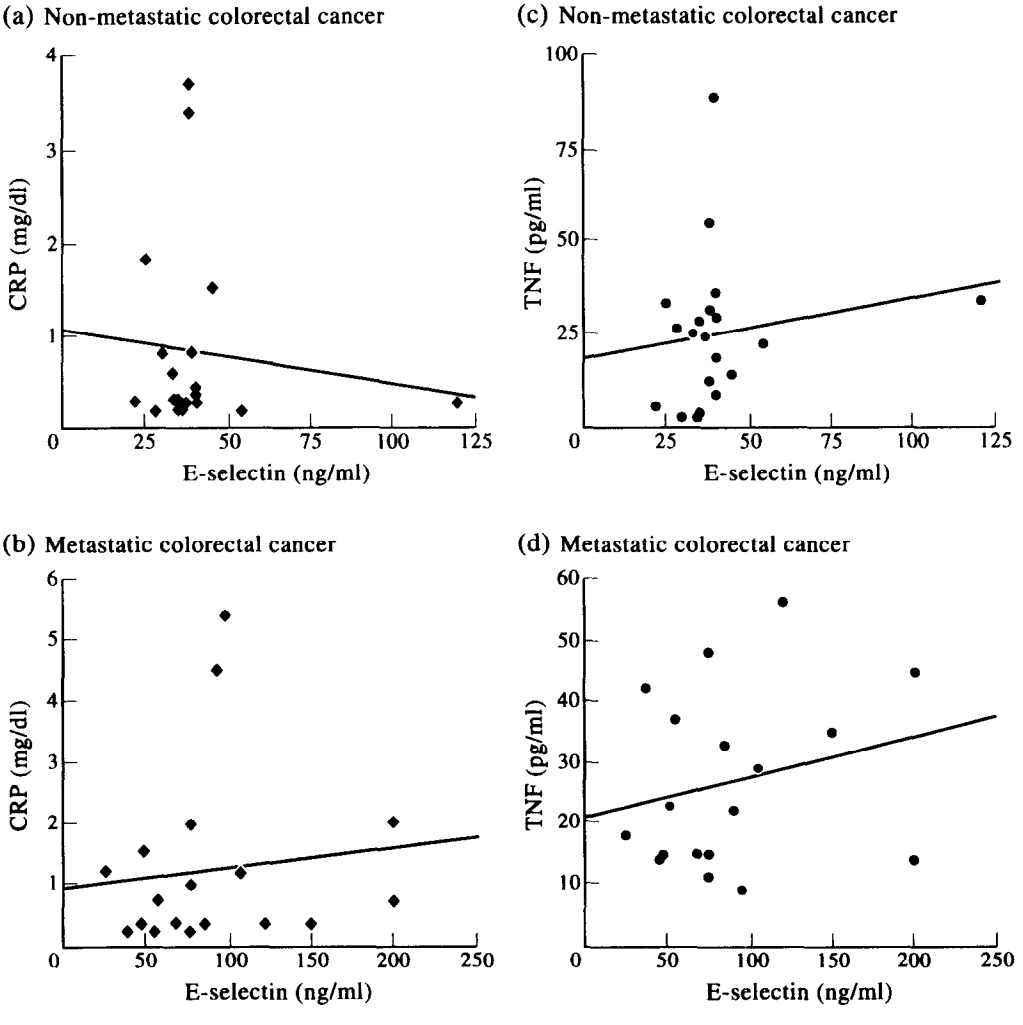

Figure 2. Correlation plots between serum E-selectin levels (ng/ml) versus C-reactive protein (CRP) levels (mg/dl) in patients with non-metastatic colorectal cancer ($n = 20$) (a) and patients with metastatic colorectal cancer ($n = 18$) (b) and between serum E-selectin (ng/ml) versus TNFα (pg/ml) in patients with non-metastatic colorectal cancer (c) and patients with metastatic colorectal cancer (d). $r = 0.0$ for all correlations.

binding to a higher degree (HL60 binding 24, 38, 39%) than the control sera from healthy individuals (HL60 binding 54, 80, 82%). This indicates that the circulating E-selectin isoform derived from tumour sera is capable of binding to HL60.

DISCUSSION

Upregulation of circulating E-selectin has been observed in a number of malignant diseases, including gastrointestinal tumours and lymphoma [13, 14]. To extend earlier findings, we analysed the level of circulating E-selectin in a patient population grouped according to metastatic and non-metastatic disease and to tumour cell morphology. We observed that elevated serum levels were exclusively associated with metastatic disease to the liver. In order to exclude that elevated E-selectin is due to inflammation in the tumour patients, serum markers of inflammation, that is C-reactive protein, fibrinogen and TNFα, were measured and did not correlate with soluble E-selectin levels.

Recent observations by Koch [15] have shown that soluble E-selectin promotes angiogenesis in rat cornea and because it is particularly elevated in diseases with vascular manifestation, e.g. the septic shock syndrome or systemic lupus erythematosus [14, 16], we propose that it might induce neovascularisation in patients with metastatic disease. Although we have shown that elevated soluble E-selectin is independent of tumour cell morphology and differentiation, we cannot completely exclude the possibility that the tumour mass or disseminated tumour growth contribute to the increased serum level of circulating E-selectin. However, 1 patient with an extraordinary large primary tumour mass did not show any elevated E-selectin level.

The molecular nature of the released E-selectin isoform and the mechanism of truncation is not yet known. Immunochemical studies suggest a lost or defective cytoplasmic domain in soluble E-selectin [16]. Therefore, we determined whether the circulating E-selectin present in the serum of colorectal cancer patients could prevent binding of HL60 cells to immobilised E-selectin. Tumour sera, more so than control sera, did inhibit E-selectin binding to HL60 cells, indicating that soluble E-selectin is capable of interacting with the ligand.

In conclusion, our study has shown that there is a marked elevation of soluble E-selectin in the serum of patients with metastatic colorectal, but not with primary tumour lesions or local recurrences of colorectal cancer. Adhesion processes of circulating tumour cells and neovascularisation in metastases might be specifically reflected by elevated serum levels of soluble E-selectin in metastasising colorectal cancer. The idea that soluble E-selectin in colorectal cancer promotes neovascularisation in metastasis is intriguing because it ties cell adhesion and angiogenesis together.

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