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Soluble CD40 Ligand Plasma Levels in Lung Cancer

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ABSTRACT

Purpose: Tumor-induced platelet activation may cause the release of various cytokines, including CD40 ligand (CD40L). Activation of the CD40/CD40L pathway in human tumors may result in thrombin generation, which is known to be involved in angiogenesis. Thus, we investigated whether soluble (s)CD40L levels are increased in patients with lung cancer as a result of platelet and/or coagulation activation.

Experimental Design: Citrated plasma samples were obtained from 120 patients with different stages and histotypes of lung cancer and 60 age- and sex-matched control subjects. sCD40L, sP-selectin (marker of platelet activation), prothrombin fragment 1 + 2, and thrombin-antithrombin III complex levels (both markers of coagulative activation) were measured in all samples.

Results: Patients with lung cancer had median sCD40L levels higher than in control subjects (0.46 versus 0.13 ng/ml; $P < 0.0001$), although correlation with the stage of disease was not evident. Nonetheless, sCD40L levels were significantly higher in squamous cancer compared with adenocarcinoma (0.75 versus 0.27 ng/ml; $P < 0.05$). Moreover, median sCD40L levels were higher in stage IV compared with nonmetastatic squamous lung cancer (1.02 versus 0.61 ng/ml; $P < 0.05$). sCD40L levels significantly correlated with sP-selectin ($P < 0.001$), prothrombin fragment 1 + 2 ($P < 0.001$), or thrombin-antithrombin III complex ($P < 0.05$) in

squamous lung cancer, but only sP-selectin ($P = 0.011$) was independently related to sCD40L.

Conclusions: These findings indicate that elevated sCD40L levels can be preferentially found in patients with advanced squamous cancer and provide evidence that increased levels of this cytokine are associated to the occurrence of *in vivo* platelet activation.

INTRODUCTION

The CD40 ligand (CD40L) is a transmembrane protein structurally related to tumor necrosis factor- α that has been first identified on CD4+ T cells (1, 2). Subsequently, CD40L has been identified on the surface membrane of activated platelets (3) from where a soluble form of CD40L (sCD40L) can be released into the circulation (4). Both membrane-bound and sCD40L interact with CD40 expressed on vascular cells, resulting in inflammatory and prothrombotic responses (3, 5). Recently, it has been suggested that activation of the CD40/CD40L pathway may enhance the procoagulant activity of tumor cells through an up-regulation of tissue factor (TF) expression, the natural activator of the extrinsic pathway of coagulation (6). Thrombin generation and peritumoral fibrin deposition are known to be involved in angiogenesis, and there is evidence that TF-mediated thrombin generation may play a predominant role in the mechanisms for tumor-induced platelet activation (7).

Platelet qualitative abnormalities have been reported in lung cancer. Several studies showed that a high percentage of lung cancer patients may have a condition of *in vivo* platelet activation, as indicated by increased levels of plasma β -thromboglobulin (8–10), serum thromboxane B₂ (10), or plasma soluble (s)P-selectin (11, 12). It is now well established that lung cancer may activate the endothelium into an adhesive, prothrombotic state (13). However, in a recent study, we did not find any association between sE-selectin (a marker of endothelial dysfunction) and sP-selectin (11), whereas the latter was significantly associated with activation markers of coagulation (12). The prothrombotic state described in cancer patients may also arise from the capacity of tumor cells to express and release procoagulant activities, *e.g.*, TF and/or cancer procoagulant (14). Both molecules are ultimately responsible for the activation of factor X, which in turn leads to the generation of active thrombin from plasma prothrombin (15). All these observations would suggest that thrombin generation caused by tumor procoagulant activities might be responsible for *in vivo* platelet activation (16) and the release of biologically active substances, as demonstrated recently for vascular endothelial growth factor (12).

In contrast to atherothrombosis, where the role of CD40/CD40L pair has been systematically investigated (17–19), its function in the biology of solid tumors remains controversial. Because it is currently estimated that >95% of plasma sCD40L is derived from platelets (20) and that approximately 50% of lung cancer patients have a condition of *in vivo* platelet activation (8–12), we can expect that >40% of lung cancer patients

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may also have increased levels of this cytokine. Therefore, the present study was designed to investigate the behavior of plasma sCD40L levels in patients with different stages of lung cancer compared with age- and sex-matched control subjects. sCD40L levels were further analyzed in association with activation markers of platelet and/or coagulation activation to test the hypothesis of an involvement of the hemostatic system. To this purpose, sP-selectin levels were used as a marker of *in vivo* platelet activation. This choice was dictated by the fact that unlike other platelet products (*i.e.*, β -thromboglobulin or serum thromboxane B2) P-selectin is particularly resistant to sampling artifacts (21). The prothrombin fragment 1 + 2 (F1 + 2) and thrombin/antithrombin complexes were analyzed as coagulation activation markers because they provide an accurate estimate of the generation of active thrombin in lung cancer (14). Finally, because it has been reported previously that patients with squamous cancer are more likely to have hemostatic abnormalities than patients with adenocarcinoma (9, 12, 22), the association among sCD40L levels, platelet and coagulation activation markers, and histological diagnosis was also analyzed.

MATERIALS AND METHODS

Patients. One hundred and twenty patients with lung cancer, treated at our institutions, entered the study. Patients (98 males, 22 females; mean age of 64.0 ± 8.4 , ranging from 35 to 82 years) were histologically diagnosed with lung adenocarcinoma ($n = 45$), squamous ($n = 55$) or other types of lung cancer, including undifferentiated ($n = 11$) and small-cell lung cancer ($n = 9$). As control group, in a 2:1 ratio, 60 control subjects matched for age and sex (49 males, 11 females; mean age of 62.7 ± 10.9 , ranging from 34 to 83 years) were also evaluated. Lung cancer was pathologically staged according to the Tumor-Node-Metastasis classification. Thirty-two (26.6%) patients were classified as stage I, 14 (11.7%) as stage II, 39 (32.5%) as stage III, and 35 (29.2%) as stage IV lung cancer. Diabetes mellitus (fasting blood glucose level >115 mg/dl or treatment with a hypoglycemic agent); body mass index >25 ; history of alcohol or drug abuse; peripheral-, cardio- and cerebro-vascular atherosclerotic diseases (by clinical history, physical examination, and instrumental diagnosis); infectious or inflammatory diseases; impaired liver (bilirubin level >1.5 mg/dl) or renal (creatinine level >1.5 mg/dl) function were considered as exclusion criteria. No patients had biopsies, chemotherapy, or immunotherapy within the 6 weeks preceding the study. No subject was on corticosteroids, nonsteroidal anti-inflammatory drugs, anticoagulant or antiplatelet agents in the two weeks preceding the study. The study was approved by the Ethical Committee of our Institutional Board, and informed consent was obtained from each subject.

Sample Collection and Immunoassays. Blood samples were withdrawn from each consenting subject, without stasis, from the antecubital vein, and anticoagulated in Na citrate 3.8% (1:9 v:v). Samples were immediately centrifuged at $1,500 \times g$ for 10 min to obtain plasma, aliquoted, coded, and stored at -40°C until the assays were performed. Plasma samples from resectable lung cancer patients were drawn within 1 week of 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.

Plasma sCD40L, sP-selectin (both by R&D Systems, Minneapolis, MN), and thrombin-antithrombin III complex (TATc; Enzygnost TAT, Dade-Behring, Marburg, Germany) levels were measured by commercially available enzyme immunoassays according to the manufacturers' instructions. F1 + 2 (Enzygnost F1 + 2; Dade-Behring) levels were measured by a commercially available enzyme immunoassay as described previously (12). 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. Statistical analysis was performed by Pearson's correlation coefficient, ANOVA and/or unpaired *t* test. When necessary, log transformation was used to normalize the data, or appropriate nonparametric tests were used (Spearman's correlation coefficient, Kruskal-Wallis method, and/or Mann-Whitney *U* test). The realized power of the study was $>90\%$. Data are presented as mean value \pm SD or median and interquartile range (25th percentile to 75th percentile). Only *P*s < 0.05 were regarded as statistically significant. All calculations were made using a computer software package (Statistica 5.5, StatSoft Inc., Tulsa, OK).

RESULTS

Plasma sCD40L, sP-selectin, F1 + 2, and TATc levels of 180 subjects, including 60 control subjects and 120 patients with histologically diagnosed lung cancer, were reported in Table 1. As shown, median plasma sCD40L ($P < 0.0001$), sP-selectin ($P < 0.0001$), F1 + 2 ($P < 0.0001$), or TATc ($P < 0.0001$) levels were significantly higher in patients with lung cancer compared with control subjects (Table 1). No differences were observed with respect to gender and age for all four variables.

Subgroup analysis of sCD40L, sP-selectin, F1 + 2, and TATc levels in patients with various histotypes of lung cancer showed that sCD40L and sP-selectin levels were increased in squamous cancer (both $P < 0.0001$) and adenocarcinoma (sCD40L, $P = 0.029$; sP-selectin, $P = 0.009$) compared with control subjects, although both variables were higher in the former compared with the latter group (sCD40L, $P = 0.02$; sP-selectin, $P = 0.03$; Table 1). No significant differences were observed between patients with undifferentiated or small-cell lung cancer and control subjects (Table 1). On the other hand, increased plasma F1 + 2 and TATc levels were found in all four groups of patients compared with controls, without any significant association with histological diagnosis (Table 1).

The analysis of the relationships among sCD40L, sP-selectin, F1 + 2, and TATc levels and clinical staging of lung cancer was carried out in patients stratified on the basis of histological diagnosis. Because of the low number of patients in the undifferentiated ($n = 11$) and small cell lung ($n = 9$) cancer groups, all further analyses were carried out on squamous ($n = 55$) and adenocarcinoma ($n = 45$) histotypes only. As shown in Table 2, median levels of sCD40L were significantly increased in stage IV squamous lung cancer ($P = 0.028$), whereas sP-selectin, F1 + 2, and TATc levels showed a trend toward increased values from stage I to stage IV squamous lung cancer, although the differences did not reach the statistical signifi-

Table 1 Subgroup analysis of plasma sCD40L,^a F1 + 2, and TATc levels in patients diagnosed with different histotypes of lung cancer and 60 age- and sex-matched control subjects

	No. cases	sCD40L (ng/ml)		sP-selectin (ng/ml)		F1 + 2 (nmol/L)		TATc (μg/l)	
		Median (IQR)	<i>P</i> ^b	Median (IQR)	<i>P</i> ^b	Median (IQR)	<i>P</i> ^b	Median (IQR)	<i>P</i> ^b
Control subjects	60	0.13 (0.05–0.44)		41.5 (31.6–61.9)		0.5 (0.4–1.4)		1.9 (1.6–5.5)	
Lung malignant									
Squamous	55	0.75 (0.37–1.45)	<0.0001	73.7 (49.7–130.0)	<0.0001	1.9 (1.3–2.1)	<0.0001	6.7 (3.4–7.4)	<0.0001
Adenocarcinoma	45	0.27 (0.15–0.66)	0.029	56.1 (39.9–88.2)	0.009	1.6 (1.0–2.1)	0.001	7.2 (4.4–7.4)	<0.0001
Undifferentiated	11	0.23 (0.06–0.27)	0.94	46.2 (38.1–67.9)	0.22	1.6 (1.1–2.2)	0.01	6.7 (5.4–7.3)	0.002
Small cell	9	0.19 (0.17–0.26)	0.38	62.3 (33.7–76.5)	0.13	1.6 (0.7–2.1)	0.01	7.2 (5.4–7.7)	0.004
Total	120	0.46 (0.18–0.96)		61.6 (41.3–102.0)		1.7 (1.0–2.1)		1.7 (1.0–2.1)	
Mann-Whitney test ^c		0.02		0.03		0.19		0.43	

^a sCD40L, soluble CD40 ligand; IQR, interquartile range; TATc, thrombin-antithrombin III complexes; F1 + 2, prothrombin fragment 1 + 2.

^b Mann-Whitney U test, control subjects vs. lung cancer.

^c Mann-Whitney U test, squamous cancer vs. adenocarcinoma.

cance. Nonetheless, median plasma levels of sCD40L (1.02 versus 0.61 ng/ml; *P* = 0.006), sP-selectin (109.2 versus 72.9 ng/ml; *P* = 0.034), F1 + 2 (2.1 versus 1.7 nmol/l; *P* = 0.044) and TATc (7.2 versus 4.1 μg/l, *P* = 0.027) were significantly higher in metastatic compared with nonmetastatic squamous lung cancer (Fig. 1). No association with the stage of disease was observed in the subgroup of patients with lung adenocarcinoma (Table 2).

Correlation analysis among sCD40L levels and all of the other variables analyzed in the overall population showed the presence of a direct correlation with sP-selectin ($r_s = 0.49$, *P* < 0.001), F1 + 2 ($r_s = 0.41$, *P* < 0.001) or TATc levels ($r_s = 0.31$, *P* = 0.013). Furthermore, sP-selectin levels significantly correlated with those of F1 + 2 ($r_s = 0.42$, *P* < 0.001) and TATc ($r_s = 0.25$, *P* = 0.034). As expected, activation markers of coagulation F1 + 2 and TATc strongly correlated between each other ($r_s = 0.54$, *P* < 0.0001). Therefore, to further analyze the relationship between sCD40L and clinical and laboratory variables of lung cancer, a multiple regression analysis including age, sex, histological diagnosis, stage, sP-selectin, F1 + 2, and TATc levels was carried out. The final model obtained by stepwise regression analysis revealed that only sP-selectin [β coefficient (SE) = 0.31 (0.12), *P* = 0.011] levels were inde-

pendently related to sCD40L (r^2 for entire model = 0.16, *P* < 0.01).

DISCUSSION

Despite recent evidence that activation of the CD40/CD40L pathway may enhance the procoagulant activity of certain tumor cells, no clinical evidence is available yet linking platelet and/or coagulation activation to sCD40L production *in vivo*. The present study is the first to demonstrate that elevated sCD40L levels can be found in patients with lung adenocarcinoma or squamous cancer compared with control subjects of similar age and gender. This study also provides evidence that increased levels of this cytokine may be at least in part related to the occurrence of *in vivo* platelet activation, as demonstrated by the significant association found between sCD40L and sP-selectin. On the other hand, no association was found between sCD40L and either tumor size or lymph node involvement, but significantly higher levels of this cytokine were found in patients with distant metastasis of squamous lung cancer.

We have shown recently that *in vivo* platelet activation results in increased levels of sP-selectin in lung cancer (11), in association with the presence of coagulation abnormalities (12),

Table 2 Subgroup analysis of plasma sP-selectin, sCD40L,^a F1 + 2 and TATc levels in patients diagnosed with lung cancer

	No. cases	sCD40L (ng/ml)		sP-selectin (ng/ml)		F1 + 2 (nmol/l)		TATc (μg/l)	
		IQR	Median	IQR	Median	IQR	Median	IQR	Median
Squamous									
I	19	0.13–1.20	0.53	41.3–101.8	63.9	0.7–2.1	0.9	2.7–7.3	3.5
II	4	0.26–0.61	0.51	48.2–91.8	66.9	1.5–1.9	1.7	2.8–4.8	3.7
III	20	0.21–1.45	0.67	43.6–130.2	77.0	1.7–2.1	2.0	3.4–7.4	7.0
IV	12	0.88–2.68	1.02	65.2–197.1	109.2	1.7–2.2	2.1	6.7–7.4	7.2
Kruskal-Wallis Anova test		<i>H</i> = 7.8, <i>P</i> = 0.028		<i>H</i> = 5.7, <i>P</i> = 0.13		<i>H</i> = 6.6, <i>P</i> = 0.09		<i>H</i> = 7.0, <i>P</i> = 0.07	
Adenocarcinoma									
I	11	0.15–0.27	0.21	39.9–87.1	55.8	1.1–2.1	1.6	7.0–7.3	7.3
II	8	0.15–0.44	0.24	39.3–57.9	49.7	0.3–1.6	1.0	2.4–7.4	5.2
III	9	0.08–0.49	0.30	41.3–56.4	49.6	0.6–2.2	1.5	1.8–7.1	3.6
IV	17	0.19–2.25	0.72	32.2–119.2	78.0	1.0–2.1	2.0	6.5–7.4	7.3
Kruskal-Wallis Anova test		<i>H</i> = 2.9, <i>P</i> = 0.41		<i>H</i> = 2.8, <i>P</i> = 0.42		<i>H</i> = 1.9, <i>P</i> = 0.59		<i>H</i> = 5.5, <i>P</i> = 0.14	

^a IQR, interquartile range; sCD40L, soluble CD40 ligand; TATc, thrombin-antithrombin III complexes; F1 + 2, prothrombin fragment 1 + 2.

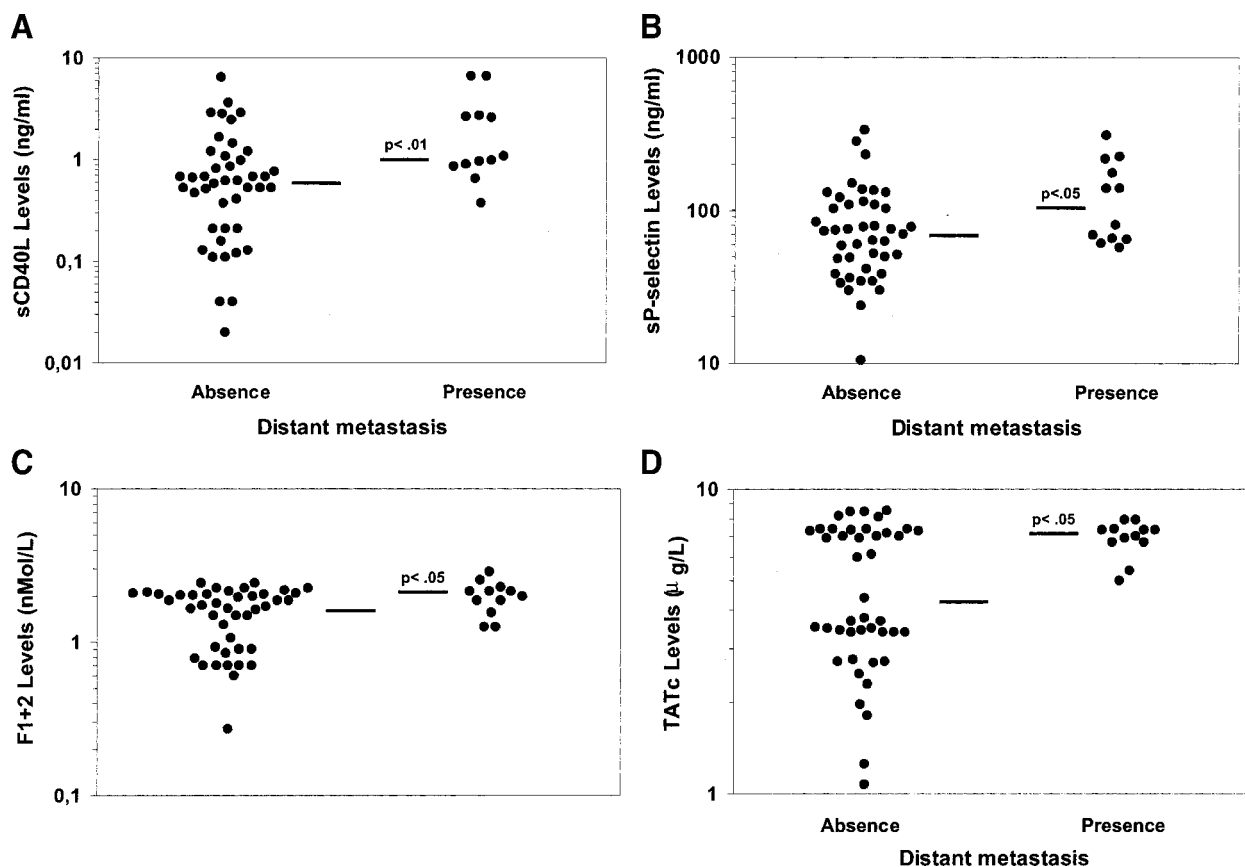


Fig. 1 Dot plot analysis of plasma soluble CD40 ligand (sCD40L; A), sP-selectin (B), prothrombin fragment 1 + 2 (F1 + 2; C), and thrombin-antithrombin complex (TATc; D) levels in 55 squamous cell lung cancer patients with or without metastatic disease. Solid lines indicates median values.

but not endothelial dysfunction (11). Preclinical and clinical studies suggested that activation of the hemostatic system may play an important role in tumor growth and metastasis. Indeed, it is well known that many human tumors possess a procoagulant activity that can be responsible for the generation of active thrombin from plasma prothrombin (15), leading to *in vivo* platelet activation (16) and release of various factors, including growth factors and cytokines, most of which may have important biological functions in the development and progression of human tumors (12, 23).

In this study, increased sCD40L showed a positive correlation with F1 + 2 and TATc, indices of *in vivo* coagulative activation. Moreover, sP-selectin correlated strongly with both F1 + 2 and TATc levels. Although it is conceivable to hypothesize that a procoagulant activity released from lung cancer cells might stimulate platelet activation and the release of sCD40L, we should also consider the possibility that platelet-released sCD40L may engage interactions with other cell types expressing CD40, including tumor-associated monocytes/macrophages and lung cancer cells (1, 24). This hypothesis is in agreement with the findings by Amirkhosravi *et al.* (6), who suggested recently that CD40/CD40L interaction through tumor cell CD40 and platelet CD40L may potentially promote clotting activation by enhancing TF expression on A375 human melanoma cells.

Of interest, increased levels of sCD40L, as well as sP-selectin and coagulation activation markers were found preferentially in squamous cell lung cancer, mainly in metastatic disease. This finding is consistent with that obtained in an immunohistochemical study of coagulation in lung cancer performed by Shoji *et al.* (25), who routinely found fibrin deposition in conjunction with TF expression in squamous cell carcinomas, whereas adenocarcinomas were relatively devoid of extravascular fibrin formation. In this light, our findings suggest that an activation of the hemostatic system in squamous cancer is likely to be responsible for the increased levels of sCD40L observed in this but not other histopathological variants of lung cancer. Furthermore, the observation that sCD40L levels were markedly elevated in patients with metastatic disease adds further evidence to the hypothesis drawn by Sabel *et al.* (24), who showed that CD40 expression by human lung cancer cells correlates with the metastatic spread of the tumor and who suggested that CD40 engagement might activate the transcription of genes involved in tumor invasion and metastasis. In this light, we should also take into consideration the findings of Melter *et al.* (26), who demonstrated that CD40/CD40L interaction promotes vascular endothelial growth factor mRNA induction in endothelial cells and monocytes as well as vascular endothelial growth factor-induced angiogenesis.

One limitation to this study derives from the fact that other cell types involved in the immune response can produce sCD40L. Therefore, it may appear questionable whether an association of sCD40L and sP-selectin is sufficient by itself to justify the conclusion of causality. However, recent findings suggest that >95% of the circulating sCD40L is derived from platelets (20) and that other sources may be negligible. All these findings add further evidence to the hypothesis drawn by Pinedo *et al.* (27) that *in vivo* platelet activation may result in the release of a wide variety of biologically active substances capable of activating other vascular cell types (*i.e.*, monocytes, endothelial cells, etc.), thus favoring the successful lodgment and extravasation of blood-borne tumor cells. Additional studies in a larger number of patients are required to better define the role of platelet-released sCD40L in human lung cancer. Better knowledge of CD40/CD40L activation in human cancer will help to improve our understanding of the pathophysiological significance of tumor-induced coagulopathies and may prompt investigators to develop novel therapeutic strategies against blood-borne metastasis.

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