Soluble Aminopeptidase N/CD13 in Malignant and Nonmalignant Effusions and Intratumoral Fluid1

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ABSTRACT

Purpose: On the basis of the finding of marked overexpression in angiogenic microvessels, aminopeptidase N/CD13 has recently been suggested to play a prominent role in tumor angiogenesis. A soluble form of CD13 (sCD13) is present in human plasma, but its role in cancer has not been addressed. We hypothesized that sCD13 would be shed by tumor cells and/or endothelial cells lining tumor vessels, giving high levels of sCD13 in intratumoral fluid (TF) deposits and in malignant effusions. If so, sCD13 could be a convenient potential marker for tumor load and/or activated tumor endothelium.

Experimental Design: We have measured the specific sCD13 activity in effusions from 90 cancer patients and 12 patients with a nonmalignant condition, and studied its relationship with other major (anti-)angiogenic factors. In a separate group of patients (n = 41), the relationship of sCD13 activity in plasma with tumor load was studied.

Results: The sCD13 activity was highest in plasma from cancer patients 71.9 (fmol/ml/s hydrolyzed substrate) versus 42.4 for healthy subjects. In TF, malignant effusions, and nonmalignant effusions, the activities were 52.8, 33.5, and 18.6, respectively. We further studied the relationship of sCD13 with tumor load as well as with vascular endothelial growth factor (VEGF), endostatin, matrix metalloproteinase (MMP)-2, MMP-9, urokinase-type plasminogen activator, and plasmin. A significant correlation of sCD13 activity in plasma was found with tumor load (r = 0.68; P = 0.01), suggesting that plasma sCD13 is, at least, partly originating from tumor-endothelium. The concentrations of VEGF and endostatin and the activities of urokinase-type plasminogen activator and MMP-9, but not MMP-2, were significantly higher in TF compared with all other effusions. In TF, a correlation between sCD13 and VEGF was found (r = 0.67; P = 0.03). No correlation of sCD13 with the other protease activities was found.

Conclusion: The sCD13 activity is elevated in plasma and effusions of cancer patients. A strong correlation of plasma sCD13 with tumor load was found. On the basis of these results, the potential of sCD13 activity as a tumor and/or angiogenesis marker warrants further investigation.

INTRODUCTION

Aminopeptidase N (APN; EC 3.4.11.2), also known as CD13 (1), is a Zn2+–dependent ectopeptidase that degrades preferentially proteins with an NH2-terminal neutral amino acid. CD13 is expressed by myeloid progenitors and monocytes, epithelial cells of the intestine and kidney, synaptic membranes in the central nervous system, fibroblasts, endothelial cells, and tumor cells (2). Known substrates of CD13 are small peptides like enkephalins, as well as cytokines such as interleukin 1B. In addition, collagen type-IV, a protein present in the basement membrane of the extracellular matrix has been suggested to be a substrate of CD13, and degradation of collagen type-IV by CD13 may contribute to the ability of tumor cells to invade the tissue and form metastases (3).

Recently, evidence for a role of CD13 in the process of tumor angiogenesis was found. Pasqualini et al. (4) demonstrated the up-regulation of CD13 expression in angiogenic vessels in MDA-MB-435 tumors in mice. In addition, angiogenic growth factors such as VEGF,3 and bFGF but also tumor necrosis factor-α and hypoxia appeared to induce CD13 expression of endothelial cells. Moreover, inhibitors of CD13 activity were able to reduce the capacity of human umbilical cord endothelial cells to form a capillary network on Matrigel (5). These results suggest that in addition to the MMPs and the plasminogen proteolytic cascade, CD13 may facilitate the degradation of the extracellular matrix.

The formation of PE and ascites is a common problem in patients with advanced cancer. The development of malignant effusions is usually a negative prognostic symptom, and is associated with a poor quality of life (6). Several factors that contribute to the formation of PE and ascites have been identified. Infiltration of tumor cells into the vasculature or into lymphatic vessels may result in obstruction (7). Additionally, angiogenic factors excreted by tumors play an important role in the formation of PE and ascites.

1 The abbreviations used are: VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; MMP, matrix metalloproteinase; PE, pleural effusion; PAI, plasminogen activator inhibitor; sCD13, soluble CD13; TF, intratumoral fluid; uPA, urokinase-type plasminogen activator; TIMP, tissue inhibitor of metalloproteinases.

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3 The abbreviations used are: VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; MMP, matrix metalloproteinase; PE, pleural effusion; PAI, plasminogen activator inhibitor; sCD13, soluble CD13; TF, intratumoral fluid; uPA, urokinase-type plasminogen activator; TIMP, tissue inhibitor of metalloproteinases.
the development of PE and ascites. One of the most important mediators of angiogenesis is VEGF. VEGF is known to stimulate the proliferation and migration of endothelial cells (8), but it also increases the permeability of vessels (9, 10). In an earlier study, we found a high concentration of VEGF in TF (11). In addition, several other studies have demonstrated elevated levels of VEGF in malignant effusions (12–14). bFGF, another stimulator of angiogenesis, has also been measured in effusions of different etiology. Probably attributable to the lack of an excretion sequence in the bFGF protein, low concentrations of bFGF have been found in effusions. No significant difference in bFGF concentration was found between malignant and benign effusions (15). In addition to growth factors, several proteases that play an important role in angiogenesis have been analyzed in effusions (16, 17). MMP-2 and MMP-9 protein concentrations were shown to be higher in malignant compared with benign effusions (18, 19). In a Phase I study with Batimastat (BB-94), a broad-spectrum MMP inhibitor, it was shown that the volume of PE decreased under treatment (20). Other studies have shown elevated levels of PAI in ovarian carcinoma cyst fluid. Moreover, PAI was found to be an independent prognostic factor in these patients; high PAI levels were correlated with an unfavorable survival (21).

Although CD13 is a membrane-bound protein, human plasma contains significant amounts of an active soluble form of CD13 (sCD13; Ref. 22, 23). This suggests that certain cells may secrete a sCD13, or that CD13 is released from the plasma membrane by shedding or by specific cleavage. In view of these findings, we hypothesized that the recently recognized angiogenic factor CD13 may be secreted from tumor cells and tumor-associated endothelial cells. If so, the level of sCD13 activity may be increased in effusions of cancer patients and, in particular, in TF. TF is a fluid phase present in the extracellular space of a tumor and is often present in certain tumor types, such as sarcoma and ovarian carcinoma. Because TF is in close contact with tumor tissue it can collect proteins that are secreted by the tumor cells and is, therefore, considered to be a reflection of the intratumoral milieu (24). In this report, we show that sCD13 activity is present in plasma, in effusions of different origin, and in TF. An elevated sCD13 activity was seen in both the effusions and the plasma of cancer patients compared with patients without a malignancy. In TF, not only sCD13 activity but also the concentration and activity of several other proteolytic enzymes, as well as the VEGF and endostatin levels, were higher than in effusions. In addition, a significant correlation between tumor load and sCD13 activity in the plasma of cancer patients was found.

**MATERIALS AND METHODS**

**Patients.** Patient characteristics are described in Table 1. PE and ascites were aspirated for diagnostic or therapeutic reasons from 102 patients, 90 of whom had a malignancy and 12 developed fluid accumulation related to an infection or congestion (see “Results”). The TF of 10 patients was obtained by aspiration of the tumor during surgical resection. TF was obtained from seven sarcomas, two ovarian carcinomas, and one teratoma. Samples of PE, ascites, or TF were centrifuged directly after aspiration at 3600 rpm/10 min/brake 9 at room temperature in a Hettich Rotanta/TR centrifuge to remove debris, and stored at −80°C until analysis. Total protein level in the samples was measured by a standard Bio-Rad assay. Heparin plasma samples of 41 cancer patients were taken for diagnostic reasons; the healthy controls were volunteers. The samples were centrifuged directly at 3000 rpm/4 min/brake 0 at 10°C, and stored at −80°C until analysis. For tumor load analysis, the cancer patients were divided into three groups: minimal disease (≤2 cm and ≤5 depositions), moderate disease (>2 cm to ≤5 cm and ≤5 depositions), and extensive disease (>5 cm or ≥5 depositions). These categories were based on computed tomography or magnetic resonance imaging scan.

**Activity of sCD13.** Activity of sCD13 in samples was measured in a 96-well plate by incubating 2 μl of sample (1:100 dilution) with 15 μM Ala-MCA (L-Alanine-4-methyl-7-coumarylamide trifluoroacetate; Fluka, Switzerland) in 10 mM HEPES-buffered PBS (pH 7.2) containing 0.1% BSA with or without the CD13-specific inhibitory antibody WM-15 (Ref. 25; 5 μg/ml; PharMingen, Heerhugowaard, the Netherlands) at 37°C. BSA (0.1%) was added to exclude effects of differences in protein concentration on sCD13 activity. The development of the fluorescent product was measured every 5 min on a Spectrafluor multiplate reader (Tecan, Salzburg, Austria; λ_exc of 360 nm, λ_em of 465 nm) for 60 min. The specific sCD13 activity was calculated from the slope of total aminopeptidase activity after subtraction of the slope of the aminopeptidase activity remaining in the presence of WM-15.

**sCD13 Protein Immunoblotting.** Protein (50 μg) of undiluted effusion sample was separated on a 6% SDS-PAGE gel, transferred to a polyvinylidene difluoride membrane (Bio-Rad Lab, Veenendaal, the Netherlands), and subjected to immunoblotting with goat polyclonal anti-CD13 (clone C-17; San-vertex, Heerhugowaard, the Netherlands) and donkey-antigoat IgG-horseradish peroxidase-conjugated (Sanvertex). Blots were developed with Enhanced Chemiluminescence (ECL Plus kit; Amersham, Roosendaal, the Netherlands).

**VEGF, bFGF, and Endostatin Concentrations.** VEGF and bFGF concentrations in PE, ascites, and TF were measured with the quantitative sandwich enzyme immunoassay, Quantikine human VEGF and Quantikine HS human bFGF, respectively (R&D Systems, Abingdon, United Kingdom), according
to the manufacturer’s instructions. Endostatin concentrations were measured by the Accucyte enzyme immunoassay, according to the manufacturer’s (Cytimmune Sciences Inc.) instructions (ITK Diagnostics, Uithoorn, The Netherlands).

MMP-2, MMP-9, uPA, and Plasmin Protein and Activity. MMP-2 and MMP-9 activity, active as well as total (latent + active), was measured as described recently (26, 27). In short, activity was measured by immobilizing MMP-2 and MMP-9 from the samples with specific monoclonal antibodies. Latent MMP-2/-9 was activated with 0.5 mM p-aminophenylmercuric acetate (APMA). Activity was measured by the addition of modified pro-urokinase and the chromogenic peptide substrate S-2444. Color development was measured at A405. Protein concentrations of MMP-2 and MMP-9 were measured with a standard quantitative ELISA, as described previously (26–28). These ELISAs recognize various forms: latent MMP, active MMP, and active MMP/TIMP complexes. Measurement of uPA protein concentration was performed using the immunoassay kit, u-PA EIA HS (Taurus, Leiden, the Netherlands). Activity of uPA and plasmin was determined as described by Koolwijk et al. (29).

Statistical Analysis. The data were analyzed using SPSS software version 8.0 (SPSS, Inc., Chicago, IL). Correlations were analyzed with the Spearman rank correlation test. For calculations of statistical differences between the different groups, the Mann-Whitney t test was used. Differences were considered significant when \( P < 0.05 \).

RESULTS

First, we identified the presence of CD13 protein in effusions by Western blot. A Western blot of sCD13 in malignant effusions of three patients is shown in Fig. 1. The apparent \( M_r \) of CD13 was 160,000, which is slightly larger than the predicted size of \( M_r \) 150,000; however, several glycosylation forms of CD13 exist, which may explain this difference (30).

sCD13 activity was present in the effusions, and malignant effusions and TF contained significantly higher levels of sCD13 activity than did the nonmalignant effusions (Fig. 2; Table 2). The level of sCD13 activity in TF (52.8 fmol/ml/s) was not statistically different from the malignant effusions. Subsequently, the nonmalignant effusions were divided in two groups according to the condition underlying the fluid formation, which were inflammation or congestion. No significant difference in sCD13 activity was found between these two groups (mean, 20.6 and 16.7 fmol/ml/s, respectively). Likewise, no significant difference was found between malignant PE and ascites (mean, 31.9 and 34.5 fmol/ml/s, respectively). Therefore, these groups were combined in the remainder of the study. No significant difference between sCD13 levels in malignant effusions in patients with different types of cancer was found (Fig. 3).

In addition, we evaluated the levels of sCD13 activity in the plasma of cancer patients and healthy controls (Table 2). We observed a marked difference in mean sCD13 activity between cancer patients (71.9 fmol/ml/s) and healthy controls (42.4 fmol/ml/s; \( P = 0.04 \)). Further analysis of the cancer patients showed a clear correlation between tumor load and sCD13 activity in plasma (\( r = 0.68; P = 0.01 \)), suggesting that the tumor environment is an important source of the sCD13 found in plasma (Fig. 4).

Next we examined the relationship between sCD13 and other (anti-) angiogenic secreted proteins. The activity of MMP-2 and MMP-9, and the fibrinolytic pathway was evaluated. The results are shown in Table 3. Because no significant difference was found between malignant PE and malignant ascites for any of the parameters studied, the results of these two groups were combined for further analysis. Active MMP-9 was present in the effusions but was found to be significantly higher in TF. No active MMP-2 could be detected, whereas the protein was present in high concentrations, with significantly higher concentrations in malignant effusions. These findings imply that most of the MMP-2 was present as MMP-2/TIMP complexes, which indicates that a high, active MMP-2 fraction, which was subsequently inactivated by TIMP (26), was present in the effusions. Except for higher uPA activity in malignant effusions and TF, the measurements of the major proteins in the fibrinolytic pathway, uPA and plasmin, did not reveal substantial differences. No significant correlation was found between the level of sCD13 activity and the MMPs or fibrinolytic enzymes.

Additionally, we measured VEGF and bFGF, two major stimulators of angiogenesis. As can be seen from Table 3, malignant effusions contained considerably higher VEGF concentrations compared with nonmalignant effusions (\( P = 0.001 \)). The concentration of bFGF was low and did not differ between the groups. Both observations are consistent with results in the literature (12–15). In TF, we found a significant correlation between sCD13 activity and the VEGF concentration (\( r = 0.67; P = 0.03 \)), supporting a causal relationship between both, as reported by Bhagwat et al. (5), who showed evidence for the up-regulation of the plasma membrane form of CD13 by VEGF. This relationship was not found in the effusions. We also measured endostatin, an endogenous potent angiogenesis inhibitor. Interestingly, we observed an increased endostatin concentration in TF (62.2 ng/ml) which was significantly higher than in nonmalignant effusions (14.1 ng/ml; \( P = 0.01 \)) or in malignant effusions (22.8 ng/ml; \( P = 0.01 \); Table 3).

DISCUSSION

CD13/Aminopeptidase N is a plasma membrane ectopeptidase that has been shown to increase the invasive capacity of tumor cells (3, 31). Recently, it was reported that CD13 might be a new marker for angiogenic vessels, by virtue of its over-expression on endothelial and perivascular cells in angiogenic tumor vessels and its role in tube formation (4, 5).

An increasing number of plasma membrane proteins, such
as VEGF receptor 1, ICAM-1, VCAM-1, and c-met are shown to have a soluble form (32–34). These proteins are produced by a separate intracellular pathway, such as alternative mRNA splicing, or by shedding or by specific cleavage from the plasma membrane. The role of the soluble forms of endothelial proteins in angiogenesis is mostly unknown, and the changes in the levels of these markers during therapy in different types of tumors remain to be assessed. The presence of sCD13 activity in human plasma has been described earlier (22, 23), but its presence or role in cancer has not been investigated thus far.

The origin of sCD13 is unknown and many cells, such as hematopoietic cells and epithelial cells, are potential sources. In this study, we hypothesized that if sCD13 activity in plasma and effusions of cancer patients was partially derived from tumor-
associated cells or activated tumor endothelial cells, this would result in higher levels of sCD13 activity in malignant effusions and, in particular, in TF compared with nonmalignant conditions.

In the present study, we show higher levels of sCD13 activity in the plasma of cancer patients than in normal plasma. In addition, our data show a strong relationship between tumor load and sCD13 activity in plasma. This new finding suggests that the tumor environment is an important source contributing to the sCD13 activity in the plasma of cancer patients. We have not yet identified the cells responsible for the production of sCD13. Tumor cells as well as tumor endothelial cells may express CD13. Because endothelial cells are in direct contact with the plasma, it is more likely that tumor-associated endothelial cells are predominantly responsible for the sCD13 in plasma. Preliminary data revealing a positive relationship of sCD13 with soluble ICAM, which is considered to be a marker for endothelial activation, support the assumption that tumor endothelial cells are an important source of the elevated sCD13 activity found in the plasma of tumor patients (data not shown).

The activity of sCD13 in malignant effusions was significantly higher than in nonmalignant conditions. Additionally, the level of sCD13 activity found in the effusions was lower than the activity in plasma. This finding is in contrast with the situation for VEGF. In patients with malignant disease, VEGF levels were much higher in effusions than in plasma (35). No significant correlation was found between VEGF levels in the effusions and in serum, which suggests that the transport of locally generated VEGF to the circulation is impaired, as suggested by Kraft et al. (35). We found the opposite for sCD13, which may suggest that in contrast to VEGF, the CD13 protein in the effusion originates from the circulation. This is further supported by the similar distribution ratio of sCD13 activity in plasma and effusions (ratio 2:1), which was found in patients both with and without the malignant disease. sCD13 in effusions is a large protein of M, 160,000, which may suggest that the passage across the endothelium is likely rather limited, although, the data concerning the dependence of vascular permeability on molecular size are scarce (36–38). Consequently, the possibility of excretion or shedding of the protein at the site of the effusions should also be considered. The presence of immune cells, such as activated lymphocytes, macrophages, monocytes and neutrophils, in effusions is of particular interest because most of the hematopoietic cells of the myeloid lineage are CD13 positive. Therefore, these cells, and the cells lining the fluids, may well be an additional source of sCD13 in the effusions.

Several studies have shown increased concentrations of proteolytic enzymes and matrix-degrading proteins in malignant effusions. These enzymes may affect the integrity of the mesothelial cell layers and the underlying basement membrane surrounding the effusion (16, 18). In this study, we examined both the concentration and the activity of two important proteolytic systems: the MMPs and the fibrinolytic pathway. Overall, we found higher concentrations of proteolytic enzymes in TF compared with other fluids. Interesting was the observation of high concentrations of MMP-2 protein, whereas we did not detect active MMP-2. These findings suggest that most of the MMP-2 protein is present in MMP-2/TIMP complexes. Active MMP-9 was present in TF, indicating a net proteolytic activity and an imbalance between MMPs and TIMPs in TF. In the effusions, no substantial MMP-9 activity was found. Our findings are in accordance with the results of Eickelberg et al. (16), who showed that MMP-2 and TIMP are constitutively expressed in effusions to function in the homeostasis of the pleura, whereas MMP-9 is induced only under specific circumstances in exudates. Because the concentration of MMP-2 antigen in effusions was significantly higher compared with MMP-9, it may be possible that preferential binding of MMP-2 to TIMPs overcomes the inhibition of MMP-9. The uPA activity was also higher in malignant conditions, in accordance with Kuhn et al. (21), who showed that high uPA is a bad prognostic factor in advanced ovarian cancer.

Of particular interest in our study is TF, which can be considered as the “conditioned medium” of the tumor (24). TF enabled us to examine more directly the relationships between tumor-secreted factors, which are not necessarily clear in more distant effusions such as PE and ascites, and certainly in the plasma. As was mentioned above, TF, compared with the effusions, contained higher levels of sCD13 activity. Interesting was
<table>
<thead>
<tr>
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<th>Nonmalignant PE/ascites</th>
<th>Malignant PE/ascites</th>
<th>TF</th>
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<tr>
<td>Protein (ng/ml)</td>
<td>27</td>
<td>35</td>
<td>50</td>
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<tr>
<td>Mean</td>
<td>0.2–56</td>
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<tr>
<td>Range</td>
<td>28</td>
<td>35</td>
<td>46</td>
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<tr>
<td>VEGF (pg/ml)</td>
<td>658</td>
<td>7,623&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18,320&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Mean</td>
<td>67–3,397</td>
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<td>698–51,452</td>
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<tr>
<td>Range</td>
<td>191</td>
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<td>15,517</td>
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<tr>
<td>bFGF (pg/ml)</td>
<td>31</td>
<td>22</td>
<td>108</td>
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<tr>
<td>Mean</td>
<td>0.4–326</td>
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</tr>
<tr>
<td>Range</td>
<td>2.3</td>
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<td>4.7</td>
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<td>14</td>
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<td>62&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Range</td>
<td>13</td>
<td>20</td>
<td>26</td>
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<td>MMP-2 Active (unit(s)/ml)</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>1,050–9,850</td>
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<tr>
<td>Range</td>
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<td>Mean</td>
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<td>818–4,433</td>
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<td>MMP-9 Active (unit(s)/ml)</td>
<td>15</td>
<td>10</td>
<td>255&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>82</td>
<td>79</td>
<td>147&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
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<tr>
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<td>55</td>
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<td>7,325&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>4,200</td>
<td>5,540</td>
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<sup>a</sup> Significantly different from nonmalignant PE/ascites.
<sup>b</sup> Significantly different from nonmalignant and malignant PE/ascites.
<sup>c</sup> Significantly different from malignant PE/ascites.
the relationship between sCD13 activity and VEGF in TF, indicating a causal relationship between expression of these two factors as described by Bhagwat et al. (5). According to these results, CD13 would be part of the protease repertoire up-regulated by VEGF in endothelial cells allowing them to degrade and invade the extracellular matrix. Interestingly, we also found the highest concentration of the antiangiogenic protein endostatin in TF, with a mean concentration of 62 ng/ml compared with 10–20 ng/ml in the plasma or serum of cancer patients (39). Therefore, this finding is a clear support for the local proteolytic production of endostatin in human tumors, as was hypothesized originally by O’Reilly et al. (40). Our results emphasize the importance of TF as a conditioned medium of the tumor, by giving a more direct insight into tumor biology, which is not necessarily reflected quantitatively in malignant effusions or in plasma from cancer patients.

In conclusion, our data show elevated activity of sCD13 in malignant effusions and in the plasma of cancer patients compared with nonmalignant effusions and plasma of healthy volunteers, respectively. The specific cells responsible for the release of sCD13 in cancer patients have to be identified. However, in plasma, we found a strong correlation with tumor load, which suggests that a considerable part of it is tumor-associated sCD13. Because tumor endothelium is in direct contact with the plasma, it may be hypothesized that activated tumor endothelial cells are an important contributing source to the tumor-associated sCD13. Moreover, the relationship of sCD13 with tumor load suggests a possible use as a general tumor marker. Additional research is needed to identify the mechanism of excretion or plasma-membrane shedding of sCD13 and to define its possible physiological function in tumor development.

REFERENCES


