Advances in Brief

Serum Endostatin Levels Are Elevated and Correlate with Serum Vascular Endothelial Growth Factor Levels in Patients with Stage IV Clear Cell Renal Cancer


Abstract

Clear cell renal carcinoma (CCRC) is a highly angiogenic tumor known to secrete vascular endothelial cell growth factor (VEGF). Endostatin is an endogenous antiangiogenic agent with antitumor activity in mice. The purpose of this study was to evaluate serum levels of endostatin in normal subjects and in patients with CCRC and to examine the relationship of these levels to circulating VEGF levels. Fifteen patients (mean age, 48 years) on a clinical protocol for stage IV CCRC at the National Cancer Institute were included in the study. Archived pre nephrectomy serum samples were analyzed for endostatin and VEGF concentrations. Endostatin and VEGF levels were compared with those of an age-matched group of volunteer blood donors (n = 18) using a competitive enzyme immunoassay. Data were analyzed using the Mann-Whitney U test and the Spearman rank correlation. Median serum endostatin levels were 24.6 ng/ml (range, 15.1–54.0 ng/ml) in CCRC patients versus 14.1 ng/ml (range, 1.0–19.3 ng/ml) in healthy controls (P < 0.0001). Median VEGF levels were 3.4 ng/ml (range, 0.1–11.2 ng/ml) and 2.5 ng/ml (range, 0.1–4.2 ng/ml), respectively (P = 0.065). A highly significant correlation was observed between endostatin and VEGF levels among the CCRC patients (r = 0.81, P = 0.0003) but not among controls (r = −0.22, P = 0.37). Endostatin levels are detectable in serum from healthy subjects as well as from CCRC patients. Levels are significantly elevated and correlate with VEGF levels in CCRC patients. Elucidating the nature of this correlation may lend insight into the regulation of tumor angiogenesis in patients with renal cancer.

Introduction

Renal cell carcinoma accounts for ~2% of all cancers, and its incidence is increasing in North America and northern Europe (1). Five-year survival rates, although gradually improving, remain around 50–60% (1) because of the high resistance of metastatic disease to systemic therapy (2). Renal cancers generally are highly vascular tumors known to secrete the proangiogenic cytokine VEGF in vitro and in vivo (3–7). Tumor VEGF expression is correlated with the severity of disease in patients with renal cell carcinoma (3, 4, 8), and some authors have suggested using circulating VEGF as a prognostic factor or tumor marker (3, 5, 9).

In addition to producing proangiogenic cytokines, recent data demonstrate that tumors can produce antiangiogenic cytokines as well (10, 11). It has been suggested that, in humans, the generation of antiangiogenic compounds in the presence of a primary tumor suppresses the growth of distant metastases (12). This phenomenon has been demonstrated in mice (11, 13, 14). However, the presence of endogenous antiangiogenic cytokines in patients with renal cell carcinoma has not been reported. In this study, we sought to determine whether circulating levels of endostatin, an antiangiogenic cleavage product of C18 (10), were elevated in patients with stage IV CCRC.

Patients and Methods

Patients. Medical records and the patient serum archives of the Surgery Branch and Urological Oncology Branch, NCI, were reviewed to identify patients who met the following criteria: (a) clinical and histological diagnosis of stage IV CCRC; (b) enrollment in an institutional review board-approved clinical protocol at the NCI; and (c) multiple aliquots of a pre nephrectomy serum sample archived. Tumor staging was based on the Robson staging system (15). Demographic and clinical data on these patients were recorded. Tumor volumes were calculated using the formula: width × length × height × π/6.

In addition, we obtained multiple aliquots of human serum from randomly selected volunteer blood donors (controls) from the Department of Transfusion Medicine, NIH. All serum samples were negative by ELISA for HIV as well as hepatitis B and C. We were provided with the age and gender of each control but received no other identifying information.

Methods. Archival serum samples collected between 1988 and 1995 from patients with CCRC who participated in an institutional review board-approved NCI study were stored in
liquid nitrogen. After retrieval from liquid nitrogen, they were stored at −80°C until ready for use. Serum samples from more recent CCRC patients and from volunteer blood donors were stored at −80°C until ready for use. Sera were thawed at room temperature, and VEGF and endostatin levels were measured using a competitive EIA (ACCUCYTE; Cytimmune Sciences, Inc., College Park, MD). Each assay was developed with its respective recombinant protein. Recombinant VEGF was obtained from PeproTech, Inc. (Princeton, NJ), whereas recombinant endostatin was obtained from EntreMed, Inc. (Rockville, MD). These recombinant proteins were the antigens for the generation of the rabbit polyclonal antiserum and were biotinylated to serve as the competitive ligands. After obtaining sufficient titers of antibody, sera were fractionated by high-performance liquid chromatography to obtain enriched immunoglobulin preparations. Briefly, the assay was run by making 4- or 5-fold dilutions of samples, which were added to a 96-well plate coated with goat antirabbit polyclonal IgG antibody. After addition of the respective competitive ligands, rabbit antihuman VEGF or endostatin polyclonal IgG antibody was added, and the plates were incubated at room temperature for 3 h. After thorough washing, streptavidin-conjugated alkaline phosphatase was added and incubated for 30 min at room temperature to dephosphorylate NADPH to NADH. After further washing, color reagents containing alcohol dehydrogenase and diaphorase were added. These reagents use NADH as a cofactor to generate formazan. Absorbances were measured at 492 nm when the A492 for the negative control well was between 1.5 and 2.0. Each sample was analyzed in triplicate, and concentrations were calculated with reference to a standard curve using Microplate Manager III (Bio-Rad, Hercules, CA). Each EIA was run blinded to the origin of the serum samples.

Rabbit antibodies against recombinant human endostatin for use in the EIA and Western blotting were generated by the method described by Shiosaka et al. (16). Briefly, 0.5 mg of recombinant human Endostatin (EntreMed, Inc., Rockville, MD) was bound to 2 ml of 32-nm colloidal gold (CytImmune Sciences, Inc., College Park, MD) at pH 8. The solution was emulsified in Freund’s Complete Adjuvant and administered s.c. into a New Zealand White rabbit. Two weeks later, the rabbit was boosted with endostatin/colloidal gold emulsified in Freund’s Incomplete Adjuvant. Six weeks after the initial immunization, a 30-ml blood sample was collected. The serum was fractionated on a Bakerbond AbX mixed ion exchange HPLC column (Baker) according to manufacturer’s specifications. Fractions corresponding to the rabbit antibody peak were pooled and dialyzed against TBS.

The ACCUCYTE endostatin EIA was validated by Western blot analysis, parallelism, quantitative recovery, and cross-reactivity studies. Additionally, intra- and interassay variability was assessed. For Western blotting, 800-µl serum samples from CCCRC patient 15 (Table 2: patient selected because of sample availability) and a healthy, 45-year-old male control were diluted 5-fold in 0.5% SDS and heated at 56°C for 5 min. The diluted samples were centrifuged in Microcon 100,000 MWCO columns (Millipore, Bedford, MA), and the filtrates were concentrated ~20-fold in Microcon 10,000 MWCO columns. Thus, serum proteins between Mw 10,000 and Mw 100,000 were concentrated ~4-fold compared with the original serum. Ten µl of each sample were run under reducing conditions on a SDS-polyacrylamide gel (NuPAGE; Novex) and transferred to a nitrocellulose membrane (Hybond; Amersham Pharmacia Biotech, Buckinghamshire, England). The membrane was incubated with 510 ng/ml purified rabbit antihuman endostatin polyclonal IgG antibody (Cytimmune Sciences), followed by horseradish peroxidase-conjugated goat antirabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Images were visualized using a chemiluminescent detection kit (ECL and Hyperfilm ECL; Amersham).

Parallelism of the EIA was evaluated using a serum and plasma sample from a normal volunteer assayed at varying dilutions. The initial dilution of the sample was 1:4. The samples were further diluted using 2-fold serial dilutions. The raw potency estimates were determined and then corrected for the dilution factor.

Quantitative recovery was assessed by adding a known amount of recombinant endostatin protein to a normal serum or plasma sample. A 0.5-ml aliquot of each sample received a 25 µl “spike” of a 2000 ng/ml stock endostatin solution, increasing the endogenous immunoreactivity by 100 ng/ml. A second and a third aliquot of the spiked and unspiked samples underwent one or two acute (i.e., within the same day) freeze (−80°C)/thaw (25°C) cycles. All samples were then analyzed by the ACCUCYTE endostatin EIA.

Cross-reactivity of the assay was tested against collagen I, collagen IV, vitronectin, fibronectin, bFGF, and Angiostatin and compared with the immunoreactivity of an equivalent amount of recombinant endostatin and heat-inactivated endostatin. To heat-inactivate endostatin, a 5 mg/ml solution of endostatin was heated to 90°C for 5 min. Each cross-reactive test molecule was run in the assay at a maximum concentration of 400 ng/ml. Two-fold serial dilutions of each test molecule to 50 ng/ml were also run to determine the specificity of any potential cross-reactivity. The potency estimates were determined based on the recombinant endostatin standard curve.

Intraassay variance was measured by analyzing three replicates of each of 26 serum samples. The mean of the individual potency estimates for each replicate was calculated, and the CV for the sample was determined. To determine interassay variation, aliquots of a single sample were analyzed over 5 days using 24 different plates.

The range of detection for the VEGF EIA was 0.195 to 50.0 ng/ml, whereas the range for the endostatin EIA was 1.95 to 500 ng/ml. Calculated concentrations exceeding the upper limit of detection for each EIA were reassayed using appropriate dilutions. Concentrations below the lower limit of detection were set at the midpoint between 0 and this lower limit (i.e., 0.098 and 0.98 ng/ml for VEGF and endostatin, respectively).

Two or three serum aliquots from the same venipuncture were analyzed for each subject. Subjects for whom only one aliquot was available were excluded from the study. The CV among the samples from each subject was calculated. For subjects from whom only two samples were available, the subject was excluded if the CV exceeded 40%. For subjects with three samples and a CV exceeding 40%, the outlying value was discarded, and the CV was recalculated using the two remaining values. If the CV still exceeded 40%, the subject was excluded.
VEGF and endostatin levels were represented as the mean of sample values considered concordant using this method.

The renal cancer cell lines 1581-RCC, 1764-RCC, UOK-125, and UOK-131 (developed in our laboratories), as well as the transformed human embryonic kidney cell 293 (American Type Culture Collection, Manassas, VA), were assayed for supernatant endostatin and VEGF concentrations. Cells were plated in 12-well plates at a density of 5 \times 10^5 cells/well in 0.5 ml of DMEM containing 10% FCS, 100 units/ml penicillin, 100 \mu g/ml streptomycin, 50 \mu g/ml gentamicin, 0.5 \mu g/ml Fungizone, and 4 \mu M glutamate (Biofluids, Rockville, MD). After 24 h incubation at 37°C, supernatants were harvested and centrifuged at 2000 \times g for 5 min. All experiments were performed in triplicate. Each sample was assayed in duplicate for endostatin and VEGF concentrations by EIA as described above.

The upper limit of normal for endostatin and VEGF levels was defined as 2 SD above the mean. Comparisons between groups were performed using the Mann-Whitney U test to compare groups according to their median values with no assumption about the scatter of the data. Correlations were performed using the Spearman rank correlation. All calculations were done using Instat 2.01 (GraphPad Software), and P < 0.05 was considered significant.

### Results

#### Demographic Data for CCRC Patients and Healthy Controls.

As described in “Patients and Methods,” subjects for whom serum endostatin and/or VEGF levels were not concordant among aliquots were excluded from the study. Of 18 patients with CCRC who met the other inclusion criteria for the study, 3 patients (17%) were excluded because of nonconcordance of values. Of 19 controls, 1 (5%) was excluded because of nonconcordance. Thus, 15 patients with CCRC and 18 controls were analyzed in this study (Table 1). Mean age was 48 years in both groups with similar age ranges. Of the 15 patients with CCRC, 12 (80%) were men, whereas 9 (50%) of the 18 controls were men.

#### Clinical Characteristics of Patients with CCRC.

Of the 15 CCRC patients, the size of the primary tumor was documented in 14 and ranged from 16 to 2246 cm^3 (median, 305 cm^3; mean, 460 cm^3; Table 2). Tumor thrombus in the renal vein was present in 3 (20%) of the 15 patients, and the inferior vena cava was uninvolved in all patients. All but one patient had solid organ metastases, with the lung being the most common site (12 of 14 patients). The remaining patient was classified as stage IV because of N2 nodal disease.

#### Serum Endostatin and VEGF Levels in Patients with CCRC and Controls.

Median serum endostatin levels were 24.6 ng/ml (range, 15.1–54.0 ng/ml) in CCRC patients and 14.1 ng/ml (range, 1.0–19.3 ng/ml) in controls (P < 0.0001; Fig. 1). Median serum VEGF levels were 3.4 ng/ml (range, 0.1–11.2 ng/ml) in CCRC patients and 2.5 ng/ml (range, 0.1–4.2 ng/ml) in controls (P = 0.065). As defined in “Patients and Methods,” the upper limits of normal for serum concentrations of endostatin and VEGF were 22.1 and 4.3 ng/ml, respectively. By these criteria, endostatin levels were abnormally elevated in 8 of 15 (53%) CCRC patients, and VEGF levels were abnormal in 6 of 15 (40%).

To determine whether the difference in gender distribution contributed to the difference in cytokine levels in the two groups, we analyzed endostatin and VEGF levels in CCRC patients and controls stratified by gender (Fig. 2). In the control group, median endostatin levels were 13.8 ng/ml (range, 11.1–15.4 ng/ml) and 15.5 ng/ml (range, 1.0–19.3 ng/ml) in men and women, respectively (P = 0.67). Median VEGF levels were 2.5 ng/ml (range, 0.3–4.2 ng/ml) and 2.4 ng/ml (range, 0.1–3.7 ng/ml) in men and women, respectively (P > 0.99). In the CCRC patients, median endostatin levels were 21.9 ng/ml (range, 15.1–54.0 ng/ml) and 40.6 ng/ml (range, 19.2–44.5 ng/ml) in men and women, respectively (P = 0.63). Median VEGF levels were 3.2 ng/ml (range, 0.1–10.8 ng/ml) and 4.8 ng/ml (range, 3.4–11.2 ng/ml) in men and women, respectively (P = 0.18).

#### Correlation between Serum Endostatin and VEGF Levels.

There was no evidence of correlation between serum endostatin and VEGF levels among the healthy controls (r = -0.22, P = 0.37; Fig. 3). There was, however, a highly significant correlation between endostatin and VEGF levels among the CCRC patients (r = 0.81, P = 0.0003; Fig. 3). Neither endostatin levels nor VEGF levels were correlated with the size of the primary tumor (r = 0.05, P = 0.82; and r = 0.26, P = 0.16, respectively; n = 14). Similarly, there was no significant difference in median levels of endostatin or VEGF in CCRC patients with or without renal vein thrombus (endostatin, P = 0.36; VEGF, P = 0.94).

#### Supernatant Concentrations of Endostatin and VEGF in Renal Carcinoma Cell Lines.

There was no detectable endostatin in the supernatants of any of the cell lines tested (Table 3). VEGF levels varied from below the limit of detection (0.195 ng/ml) to 7.3 ng/ml. Neither endostatin nor VEGF was detectable in the supernatant of 293 cells.

#### Validation of ACCUCYTE Endostatin Competitive Enzyme Immunoassay.

Western blot analysis of the serum from patient 15 (endostatin concentration, 44.3 ng/ml by EIA) revealed a band of endogenous endostatin immunoreactivity with mobility equivalent to that of recombinant human endostatin (Fig. 4). This band was not visualized in the lane containing the normal human serum sample (endostatin concentration, 13.4 ng/ml by EIA).

The validation data derived from studies of parallelism are presented Fig. 5. Dilution of both the serum and plasma sample led to diminished endostatin immunoreactivity paralleling the dilution curve of the recombinant endostatin standard. Quantitative recovery validation of the ACCUCYTE endostatin EIA yielded recovery of the spike ranging from 83 to 96%, with an average recovery of 90%. Of the molecules tested (collagen I, collagen IV, vitronectin, fibronectin, basic fibroblast growth factor, and angiostatin), angiostatin had the highest cross-reactivity, which was 0.04% as compared with an equivalent dose of

| Table 1 Demographic data for CCRC patients and healthy controls |
|------------------|------------------|-----------------|-----------------|
|                  | n                | Mean age (range) | Male (%) Female (%) |
| CCRC             | 15               | 48 (26–69)       | 12 (80) 3 (20)     |
| Control          | 18               | 48 (26–68)       | 9 (50) 9 (50)      |

* In years.
endostatin. Heat-inactivated endostatin retained 1% immunoreactivity. No visible precipitates were observed in the boiled preparation; therefore, the diminished immunoreactivity of heat-inactivated protein did not appear related to a change in solubility. Under less damaging conditions, such as preparing the molecule for Western blot analysis under reducing conditions, the endostatin retained its immunoreactivity (data not shown; the endostatin was not heated for the Western blot in Fig. 4).

Intraassay evaluation of three replicates of each of 26 serum samples yielded an average CV of 6.4%. Evaluation of interassay variation yielded a SD of 0.83 ng/ml and a CV of 13%.

Discussion

In this study, we demonstrated that serum endostatin levels are elevated in the majority of patients with stage IV CCRC. Endostatin is one of a number of endogenously generated angiostatic protein fragments that have been shown to have antitumor activity in murine models (10). Endostatin originally was identified in the supernatant of the murine hemangioendothelioma cell line, EOMA. Interestingly, we are not aware of any other murine or human tumor cell lines that produce measurable levels of endostatin in their supernatants (10). This observation includes the evaluation of renal cancer cell lines in the present study.

The nucleotide sequence encoding endostatin resides within the COOH-terminal, noncollagenous domain of C18, termed NC1 (10). C18 has been shown to be a member of the unique collagen family, the multiplexins, that resides in base-
ment membranes and particularly in the liver (17–19). Most collagens undergo cleavage of their noncollagenous domains after secretion from the cell (19, 20). This phenomenon would explain the lack of endostatin secretion from most tumor cell lines in vitro, including those from renal cell carcinomas. More likely, endostatin is cleaved from C18 extracellularly. Recently, elastase and cathepsin activities have been shown to cleave endostatin from NC1 in vitro (21, 22). The mechanisms of endostatin generation in vivo remain unknown.

Our findings suggest that endostatin is detectable in healthy subjects. The validation data evaluating parallelism and quantitative recovery using the ACCUCYTE endostatin EIA show that the immunoreactivity detected in normal human serum and plasma is essentially identical to that of recombinant endostatin. The absence of a Mr 20,000 endostatin-immunoreactive band Western blotting of normal human serum was to be expected, because the 4-fold concentration of serum samples would yield an estimated endostatin concentration (53.6 ng/ml) below the sensitivity of the blot (125 ng/ml). Endostatin in the circulation of healthy subjects may play a role in the homeostatic regulatory network controlling angiogenesis, termed the “angiogenic switch” (23–25). Alternatively, it may be generated as a by-product of physiological collagen turnover.

Western blotting of the concentrated serum sample from CCRC patient 15 (calculated endostatin level after concentration, 177.2 ng/ml), however, revealed a band with mobility equal to that of recombinant endostatin (Fig. 4). We believe that the larger, ill-defined areas of intensity on the Western blot are most likely attributable to nonspecific binding to ubiquitous serum proteins. John et al. (26) have reported the presence in human plasma of a number of endostatin antibody-immunoreactive protein fragments; however, these are relatively small molecules with molecular weights ranging from Mr 16,000 to Mr 22,000.

In this study, patients with CCRC had higher serum VEGF levels than healthy controls, although this difference was not quite significant. Given previous data in the literature with larger numbers of patients (3), we suspect that the high VEGF levels seen in our series represent a real phenomenon. Nonetheless, the difference in endostatin levels between CCRC patients and controls was highly significant. Elevations in circulating endostatin levels are not unique to patients with cancer. Perturbed regulation of angiogenesis is an important feature of rheumatoid, vascular, and other nonneoplastic diseases (25); recently, circulating endostatin levels were reported to be elevated in patients with systemic sclerosis (27).

Most importantly, serum endostatin levels correlated significantly with serum VEGF levels in patients with CCRC but...
The angiogenic phenotype of the tumor and the relatively low response to circulating endostatin levels in CCRC, because of involvement of murine endostatin for inhibition of endothelial cell proliferation is ~100 ng/ml (10, 28) and may be much higher for human endostatin (29). Furthermore, our data indicate that VEGF is secreted by at least some renal cancer cell lines in vitro in the absence of endostatin. Finally, in a murine model (28), adenoviral delivery of the endostatin gene leads to high plasma concentrations of endostatin (mean, 1770 ng/ml) without a concomitant elevation in VEGF levels (data not shown).

We therefore have proposed two hypotheses to explain the correlation of endostatin and VEGF in this study, summarized in Fig. 6. In addition to VEGF, invasive tumors secrete multiple collagenases, including matrix metalloproteinases, which facilitate digestion of the extracellular matrix and basement membrane, allowing the tumor access to the circulation. This is true of renal carcinomas as well (30, 31). One of these collagenases may cleave endostatin from C18 (Fig. 6A). Evolution may have favored the development of cleavage products that are opposite in activity to the process which created them to keep such pathological processes in check or as part of the “off” switch that controls physiological angiogenesis. The relationships between PEX and matrix metalloproteinase-2 and between angiostatin and macrophage-derived metalloelastase may be other examples of this phenomenon (32, 33).

VEGF, in addition to promoting EC proliferation, also induces the release of multiple collagenases and other proteases from ECs (34–36). Therefore, the collagenase that cleaves endostatin from C18 in vivo may also be EC derived and its expression up-regulated by VEGF binding to its EC receptors (Fig. 6B). We believe that both of these processes may occur simultaneously, because tumor neoangiogenesis is known to involve degradation of extracellular matrix components by both endothelial cells and tumor cells. The expanding list of endogenous pro- and antiangiogenic factors reveals the complexity of the homeostatic system controlling angiogenesis.

In conclusion, serum endostatin levels can be reliably determined using a competitive enzyme immunoassay, are detectable in normal subjects, and are elevated in patients with CCRC. Furthermore, endostatin levels are significantly correlated with VEGF levels in CCRC patients but not in healthy controls. We hypothesize that elevated endostatin levels represent an attempt at a compensatory response to the angiogenic phenotype of CCRC. We are currently investigating the interrelationship of endostatin, VEGF, and other related cytokines in vitro and in vivo animal models, as well as in patients with other tumor histologies. We believe that elucidating the nature of the homeostatic relationship between pro- and antiangiogenic, tumor-derived cytokines will be critical in developing and refining treatment strategies to inhibit tumor angiogenesis.

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References


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