Quantitative Correlation of Serum Levels and Tumor Expression of Vascular Endothelial Growth Factor in Patients with Hepatocellular Carcinoma

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ABSTRACT

Recent studies have suggested that serum levels of vascular endothelial growth factor (VEGF) may provide useful prognostic information in patients with various types of cancers. However, there has been a debate on whether serum VEGF level is a true reflection of tumor angiogenic activity in cancer patients. This debate originates from the finding that most VEGF in the serum is released from platelets during clotting. It has been postulated that platelet may serve the role of storage for circulating VEGF derived from the tumors. We conducted a study to clarify whether the platelet load of VEGF in the circulation correlates with tumor expression of VEGF. We measured quantitatively the serum VEGF levels and tumor cytosolic VEGF concentration by an ELISA and tumor VEGF mRNA by real-time quantitative reverse transcription-PCR in 60 patients with hepatocellular carcinoma. Serum VEGF levels correlated significantly with platelet counts (r = 0.662, P < 0.001). When corrected for platelet count, serum VEGF/platelet correlated significantly with tumor cytosolic VEGF concentration (r = 0.447, P = 0.006), which in turn correlated with VEGF mRNA expression in the tumors (r = 0.315, P = 0.020). Advancing tumor stage was associated with a significant increase in tumor cytosolic VEGF concentration (P = 0.006), tumor VEGF mRNA expression (P = 0.012), serum VEGF/platelet (P = 0.001), and serum VEGF165 levels (P = 0.003). In conclusion, our data showed that the platelet load of VEGF in the circulation correlated positively with tumor VEGF expression. This study provides strong evidence that supports the use of serum VEGF level as an indirect estimate of tumor VEGF expression.

INTRODUCTION

Tumor angiogenesis is fundamental to tumor growth and metastasis (1). VEGF is a potent angiogenic factor with five molecular isoforms generated by alternative splicing of VEGF mRNA, which are composed of 206, 189, 165, 145, and 121 amino acid residues, respectively (2). VEGF121 and VEGF165 are both secreted into the circulation, but VEGF145 is the predominant isoform secreted by most tumors (2). The other isoforms do not enter the circulation in a significant amount because they are either bound to the extracellular matrix (VEGF145) or not secreted (VEGF189 and VEGF206).

Numerous studies have shown that high-serum VEGF levels predict advanced disease in cancer patients (3–6). Serum VEGF may be a surrogate marker of tumor angiogenesis (7). However, there has been a debate of whether serum VEGF level actually reflects tumor expression of VEGF (7–12). Megakaryocytes are known to express VEGF, which is stored in the α granules of the platelets and released during platelet activation (13, 14). Comparison of serum and plasma VEGF levels in the same cancer patient has shown much higher serum levels (8, 15), suggesting that most VEGF in the serum is derived from the platelets during clotting (16). Furthermore, a significant correlation between serum VEGF and platelet count has been observed in cancer patients (9, 17). Some authors suggested that serum VEGF might reflect platelet count rather than tumor VEGF secretion (16). Others advocated that platelet may store VEGF secreted from the tumor based on the finding of several-folded higher cellular content of VEGF in platelets of cancer patients compared with healthy individuals (9, 17, 18). A recent study further demonstrated that serum VEGF levels corrected for variation in platelet counts correlated with advancing tumor stage, and the authors recommended serum VEGF normalized by platelet count as a standardized measurement of circulating VEGF (11). However, there is no evidence in the literature of a direct quantitative correlation between the levels of circulating VEGF and tumor expression of VEGF.

HCC is a hypervascular tumor. Angiogenesis plays a significant role in the progression of HCC (19, 20). VEGF is an important mediator of angiogenesis in HCC (21, 22). Serum VEGF level has been found to predict venous invasion and metastasis in HCC (23, 24). To test the hypothesis that serum VEGF levels reflect tumor VEGF expression, we conducted a study to quantitatively evaluate the relationship between serum VEGF levels and tumor VEGF expression in patients with HCC.

MATERIALS AND METHODS

Patients and Specimens. Between 1998 and 2001, 60 patients (44 men and 16 women, age 16–79 years) undergoing resection of HCC were recruited in this study with informed consents. The study protocol was approved by the Ethics Committee of our institution. Serum hepatitis B surface antigen was positive in 47 patients (78%). No patient had received any anticancer therapy before surgery. Tumor was staged according to the Tumor-Node-Metastasis staging (25).

Before surgery, peripheral venous blood samples were taken from the patients into a serum separator tube and centrifuged at 3000 rpm for 10 min, then stored at −70°C. Fresh tumor tissue and nontumorous liver were obtained immediately after tumor resections.

Quantitation of Serum and Tumor Cytosolic VEGF Concentration. VEGF165 of both serum and tissue cytosolic samples were quantified by Quantikine human VEGF Immunosorbent assay kit (R&D Systems, Minneapolis, MN). The assay had been shown to be reproducible (4, 6, 14). It exhibited no significant cross-reactivity with other angiogenic factors and had a sensitivity of 9 pg/ml. All samples were assayed in duplicate. To correct for variation in platelet counts, VEGF per platelet (pg/10⁶ platelets) was calculated by dividing serum VEGF concentration (picogram/milliliter) by the platelet count (×10⁹/ml; Refs. 10, 11, and 17).

Protein cytosolic fraction was obtained by homogenization of tissues as described (26). Homogenates were centrifuged at 20,000×g at 4°C for 10 min. The supernatants were collected for assay of the cytosolic VEGF165 concentration, and the total protein concentration was determined using Bio-Rad total protein assay system (Bradford, Hercules, CA).

Immunohistochemical Staining for Tumor VEGF165 Expression. Paraffin-embedded, 4-μm sections were deparaffinized, rehydrated, treated with 3% H₂O₂ in methanol, and then microwaved for 10 min. Endogenous biotin was blocked. Sections were then incubated with normal rabbit serum for 30 min at 37°C. VEGF165 mouse antihuman monoclonal antibody (R&D Systems) at 1:200 dilution was applied, and sections were then incubated with 1:100 rabbit antimouse biotinylated IgG secondary antibody (DAKO Corp., Carpinteria, CA) for 1 h at 37°C. Finally, sections were incubated with 1:100 avidin-biotin complex method-horseradish peroxidase (DAKO) and developed...
with 3,3'-diamino-benzidine tetrachloride (DAKO). VEGF_{165} expression was classified as strong if >30% of cells were stained positive and weak if <30% cells were stained positive.

Real-time Quantitative Reverse transcription-PCR for Tumor VEGF_{165} mRNA Expression. Total RNA from tumor and nontumor frozen tissues were extracted using RNasy Mini Kit (Qiagen, Valencia, CA). RNA was pretreated with DNase I (Invitrogen, Carlsbad, CA), and cDNA was synthesized by SuperScript (Invitrogen). The VEGF_{165} probe (5'-6Fam-TGA ATG CAG ACC AAA GAA AGA TAG AGC AAG-TAMRA-3'), forward primer (5'-AGC TTC CTA CAG CAC AAC AAA TG-3'), and reverse primer (5'-CAA GGC CCA CAG GGA TTT T-3') were designed using the ABI PRISM Primer Express Software (Applied Biosystems, Foster City, CA). Real-time quantitative PCR analysis was performed as described (27) using ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Ribosomal 18 s was used as an internal control.

Calculation of Real-time Quantitative Reverse Transcription-PCR Data. The relative amount of VEGF_{165} mRNA, normalized to an internal control ribosomal 18 s and relative to a calibrator, was given by Livak and Schmittgen (28):

\[2^{-\Delta \Delta CT}\]

where

\[\Delta \Delta CT = (C_T, VEGF_{165} - C_T, r18s)_{sample} - (C_T, VEGF_{165} - C_T, r18s)_{calibrator}\]

Fig. 1, A and B are the amplification plots of VEGF_{165} and ribosomal 18 s, respectively, in which changes in normalized reporter signal (ΔRn, Y axis) are plotted against cycle number (X axis). C_{T} represents the fractional cycle number at which there is a significant increase in Rn above the chosen threshold (horizontal black line). B, amplification plots of ribosomal 18 s (multiplex) in different tissue samples. Emission intensity of the reporter (VIC) dye (ΔRn, Y axis) versus cycle number (X axis).
the calibrator to compare the relative amount of target in different samples and adjust for the plate-to-plate variation in amplification efficiency.

Statistical Analysis. Continuous data are presented as median value (interquartile range). The Kruskal-Wallis, Mann-Whitney U, and Wilcoxon rank tests were used to evaluate differences between multiple groups, unpaired and paired observations. Correlations were evaluated using the Spearman rank test. Statistical significance was taken as $P < 0.05$. All statistical analyses were performed using a statistical software package (SPSS, Inc., Chicago, IL).

RESULTS

Serum VEGF$_{165}$ Level. The median serum VEGF$_{165}$ level among the 60 patients was 248 pg/ml (140–425). The median platelet count was $162 \times 10^9$/ml (133–235). The serum VEGF$_{165}$ levels correlated significantly with platelet counts ($r = 0.662, P < 0.001$; Fig. 2). The median serum VEGF$_{165}$/platelet was 1.4 pg/10$^9$ platelets (0.75–2.44).

The median tumor size was 7 cm (3.5–9.5). Both the serum VEGF$_{165}$ levels ($r = 0.468, P < 0.001$) and platelet counts ($r = 0.523, P < 0.001$) correlated significantly with tumor size. However, serum VEGF$_{165}$/platelet was not related to tumor size ($r = 0.134, P = 0.307$). Platelet counts, serum VEGF$_{165}$ levels, and serum VEGF$_{165}$/platelet showed significant increase with advancing tumor stage (Figs. 3 and 4). A high-serum VEGF$_{165}$/platelet was significantly associated with macroscopic tumor invasion of intrahepatic venous branch ($P = 0.036$). There were no significant differences in serum VEGF$_{165}$ levels ($P = 0.214$) and serum VEGF$_{165}$/platelet ($P = 0.776$) between cirrhotic ($n = 25$) and noncirrhotic patients ($n = 35$).

Tumor VEGF$_{165}$ Expression. The cytosolic VEGF$_{165}$ concentration in the tumors (median 46.3 pg/mg total protein, 23–143.6) was significantly higher than that in the nontumorous livers (median 22.8 pg/mg total protein, 12.7–37.6; $P < 0.001$). Immunohistochemical study showed staining of VEGF$_{165}$ in the tumor cells to a variable extent in all tumors. VEGF$_{165}$ was expressed predominantly in the cytoplasm of the tumor cells (Fig. 5). Little staining was found in the stromal cells. Tumors with strong VEGF$_{165}$ staining had significantly higher cytosolic VEGF$_{165}$ than those with weak VEGF$_{165}$ staining (median 68.4 versus 38.5 pg/mg total protein, $P < 0.001$). Staining for VEGF$_{165}$ was also observed in the hepatocytes in nontumorous liver sections, but the majority of cases ($n = 62, 87\%$) expressed only weak...
staining. In contrast, among the 60 tumors, 32 cases (53%) expressed strong VEGF165 staining.

The relative tumor VEGF165 mRNA (median 3.44, 1.94–7.12) was higher than the relative nontumorous VEGF165 mRNA (median 1.72, 0.94–2.9; \( P < 0.001 \)). There was a significant correlation between VEGF165 mRNA and cytosolic VEGF165 protein in both the tumors (\( r = 0.315, P = 0.02 \)) and nontumorous livers (\( r = 0.34, P = 0.009 \)).

There was no significant correlation between tumor cytosolic VEGF165 and tumor size (\( r = 0.151, P = 0.215 \)), or between tumor VEGF165 mRNA and tumor size (\( r = 0.118, P = 0.283 \)). However, both tumor cytosolic VEGF165 (\( P = 0.006 \)) and VEGF165 mRNA (\( P = 0.012 \)) increased with advancing tumor stage. High tumor cytosolic VEGF165 (\( P = 0.042 \)) and VEGF165 mRNA (\( P = 0.047 \)) were also associated with tumor invasion of venous branch.

**Correlation between Serum VEGF Level and Tumor Expression of VEGF.** A trend toward a positive correlation between serum VEGF165 and tumor cytosolic VEGF165 was observed (\( r = 0.277, P = 0.103 \)). Serum VEGF165/platelet was significantly correlated with tumor cytosolic VEGF165 (\( r = 0.447, P = 0.006 \); Fig. 6). There was no significant correlation of nontumorous cytosolic VEGF165 with either serum VEGF levels (\( r = 0.111, P = 0.407 \)) or serum VEGF165/platelet (\( r = 0.021, P = 0.878 \)). Strong tumor immunostaining of VEGF165 was also associated with higher serum VEGF165/platelet compared with weak VEGF165 staining (median 1.75 versus 0.95/10⁶ platelets, \( P = 0.019 \)). Serum VEGF165/platelet also correlated with tumor VEGF165 mRNA (\( r = 0.342, P = 0.023 \)).

**DISCUSSION**

One of the potential clinical implications of tumor angiogenesis is its prognostic value in cancer patients (29). In this respect, measurement of circulating levels of angiogenic factors has several advantages over direct assessment of tumor angiogenesis: (a) it does not require a tumor specimen and thus is theoretically applicable to every cancer patient; (b) it is technically simpler; and (c) repeated measurements can be made in the same patient before and after anticancer treatments. Most studies showed that serum VEGF has a prognostic value in various cancers (7). However, the unsettled issue of whether serum VEGF level can reflect tumor VEGF expression has become a major obstacle in its clinical application.

Some indirect evidence has suggested that the tumor is a major source of serum VEGF in cancer patients. A remarkable decrease in serum VEGF levels after tumor resection has been reported (6, 30). One study demonstrated that serum VEGF levels in mesenteric venous blood draining from the tumors were several-fold higher compared with peripheral blood in colorectal cancer patients, indicating secretion of VEGF from the tumors into the circulation (31). However, a more recent study found no significant increase in serum or plasma VEGF levels in the vein draining the tumors in patients with various carcinomas (32). To validate the use of serum VEGF as an
estimate of tumor angiogenic activity, it is imperative to show a direct quantitative correlation between serum VEGF level and tumor expression of VEGF.

In agreement with previous studies (9, 11, 17, 23), our data showed that serum VEGF_{165} levels correlated with platelet counts. Although platelet is known to express VEGF (13, 14), several studies suggested that platelet probably has a more important role as a scavenger of the VEGF in the circulation. Although there was a significant correlation between serum VEGF and platelet count in cancer patients, such a correlation was not observed in healthy controls (10). Furthermore, George et al. (11) showed that serum VEGF levels correlated with platelet counts in patients with colorectal cancer but not in patients with benign colorectal disease. Platelet is known to endocytose and store various circulating proteins (33). The scavenger role of platelets may have a physiological function of restricting the angiogenic activity of circulating VEGF to sites where coagulation takes place, such as a healing wound. The leaky tumor vasculature may allow platelets to come into contact with the tumor and deposit VEGF to enhance tumor angiogenesis (34). VEGF is known to stimulate the recruitment of endothelial progenitors from the bone marrow for physiological vasculogenesis (35), which can be damped by the storage of VEGF in platelets. By storing VEGF, platelets may have a net proangiogenic effect on tumor endothelium (36). Our study shows that serum VEGF_{165}/platelet correlated with tumor VEGF_{165} protein level. This provides the most direct evidence thus far available for the role of platelet in the storage of VEGF released from the tumors.

We also evaluated the tumor expression of VEGF_{165} at the transcriptional level using real-time quantitative reverse transcription-PCR. We demonstrated a significantly higher VEGF_{165} mRNA expression in the tumors than in the nontumorous liver. VEGF_{165} mRNA expression correlated significantly with VEGF_{165} protein expression in the tumors. Both tumor cytosolic VEGF_{165} protein and VEGF_{165} mRNA increased significantly with advancing tumor stage. Serum VEGF_{165}/platelet also increased with advancing tumor stage. Neither serum VEGF_{165}/platelet nor tumor VEGF_{165} expression was significantly related to tumor size. However, both serum VEGF_{165}/platelet and tumor VEGF_{165} expression correlated significantly with venous invasion by the tumor, which is another determinant of tumor stage apart from tumor size.

Because the platelet load of VEGF correlates with tumor VEGF expression, it is important to include the VEGF stored in the platelets in the measurement of circulating VEGF as an indirect estimate of tumor angiogenic activity. Our data support the use of serum VEGF level instead of plasma VEGF level in the evaluation of circulating VEGF. In an unpublished study by the authors, high pretreatment serum VEGF levels were found to predict poor prognosis in patients undergoing transarterial chemoembolization for HCC. Another group has demonstrated an increase in serum VEGF levels after transarterial embolization of HCC (37). These data strengthen the clinical significance of serum VEGF in HCC patients. Because VEGF is a key mediator of angiogenesis secreted by most cancers (2), it is reasonable to speculate that the results from the current study can be generalized to other common human cancers, but this needs to be confirmed with additional studies. A recent study that measured matched preoperative plasma and serum VEGF levels in patients with colorectal cancer showed that high-serum VEGF level but not plasma VEGF level was an independent predictor of survival (38). It is unclear whether total serum VEGF level serum VEGF/platelet provides better prognostic prediction. In our study, advanced tumor stage was associated with an increased platelet count. A study in colorectal cancer has demonstrated that thrombocytosis is associated with more advanced disease and worse prognosis (39). Serum VEGF level reflects both the platelet load of VEGF and the platelet count; thus, it may be a better prognostic marker than serum VEGF/platelet. Some recent studies suggested that serum VEGF is not all tumor derived, and other cytokines secreted by the tumors, such as interleukin-6, may up-regulate the expression of VEGF in the platelet (17, 32). The relative contribution of VEGF directly secreted from the tumor and VEGF expressed by the platelet itself to the platelet load of VEGF in cancer patients need clarification with additional studies.

In conclusion, this study demonstrated for the first time that the platelet load of VEGF in the circulation of cancer patients correlated quantitatively with the expression of VEGF in the tumors. Our study provides strong evidence that supports the use of serum VEGF level as an indirect estimate of tumor VEGF expression.

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