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Post-collection, pre-measurement variables affecting VEGF levels in urine biospecimens

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Abstract

Angiogenesis, the development and recruitment of new blood vessels, plays an important role in tumour growth and metastasis. Vascular endothelial growth factor (VEGF) is an important stimulator of angiogenesis. Circulating and urinary VEGF levels have been suggested as clinically useful predictors of tumour behaviour, and investigations into these associations are ongoing. Despite recent interest in measuring VEGF levels in patients, little is known about the factors that influence VEGF levels in biospecimens. To begin to address this question, urine samples were collected from patients with solid tumours undergoing radiotherapy and healthy volunteers. Four factors were examined for their effects on VEGF concentrations as measured by chemiluminescent immunoassay: time from sample collection to freezing, number of specimen freeze–thaw cycles, specimen storage tube type and the inclusion or exclusion of urinary sediment. The results of this study indicate that time to freeze up to 4 hrs, number of freeze–thaw cycles between one and five, and different types of polypropylene tubes did not have statistically significant effects on measured urinary VEGF levels. Urinary sediment had higher VEGF levels than supernatant in five of six samples from healthy patients. It is not clear whether there is an active agent in the sediment causing this increase or if the sediment particles themselves are affecting the accuracy of the assay. Therefore, we recommend centrifuging urine, isolating the supernatant, and freezing the sample in polypropylene microcentrifuge tubes or cryogenic vials within 4 hrs of collection. In addition, we recommend the use of samples within five freeze–thaw cycles.

Keywords: angiogenesis • VEGF • tumour markers • urine • biospecimens

Introduction

Vascular endothelial growth factor (VEGF) is an important stimulator of angiogenesis, the development and recruitment of new blood vessels. Through angiogenesis, VEGF plays an important role in the pathogenesis of cancer, as growing tumours require new conduits to provide nutrients and remove waste. In addition, VEGF increases vascular permeability, which may facilitate metastasis and result in increased oxygen delivery to tumours. VEGF is expressed by a variety of solid and haematologic neoplasms [1] and inhibition of VEGF is used therapeutically in the treatment of certain cancers.

A variety of histology-specific tumour markers are used clinically. For example, prostate-specific antigen (PSA) is used in the diagnosis and management of prostate cancer. However, VEGF has the potential to serve as a more general tumour marker, with prognostic

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value across an otherwise heterogeneous group of cancers. The role of VEGF as a clinically useful predictor of tumour behaviour is currently under investigation. In a literature review, Poon *et al*. cited evidence for correlations between circulating levels of VEGF and the endpoints of tumour progression or patient survival for breast [2, 3], lung [4, 5], colorectal [6–11], gastric [12–14], hepatocellular [15–17], prostate [18, 19], bladder [20], renal cell [21–23], ovarian [24–26], nasopharyngeal [27], and haematologic [28–30] malignancies [31]. In addition, VEGF gene polymorphisms may affect the prognosis for certain cancers [32].

Measuring VEGF in the urine, as opposed to serum or plasma, is preferable since urine sample collection is less invasive than drawing blood. In addition, venipuncture activates platelets and may release cytokines, including VEGF, artificially elevating measured VEGF levels [33, 34]. The prognostic value of urinary levels of VEGF has been a subject of recent investigation. Chan *et al*. [35] evaluated urinary VEGF levels in patients undergoing radiotherapy and reported that levels at presentation were significantly different between patients with local-regional cancer and normal controls, as well as between patients with metastatic prostate cancer and local-regional disease. A difference was also found between patients with no evidence of disease after radiation and patients with persistent or recurrent disease following radiotherapy.

Despite recent interest in measuring urinary VEGF levels in patients, relatively little is known about the non-patient factors that influence VEGF levels in patient biospecimens. In this study, several variables that could affect VEGF levels in human urine specimens were examined. The focus was narrowed to variables that could be controlled after the sample is collected, but before it is thawed for measurement. For more information on the variables affecting the measurement process itself, see Kirk *et al*., also in this issue.

The first factor examined was the time delay between specimen collection and sample freezing for storage. We hypothesized that proteases in the urine degrade VEGF over time and that the VEGF levels would decline when the urine was left at room temperature for extended periods before freezing.

Next, the effect of freeze–thaw cycles on VEGF levels was examined. Schaub *et al*. evaluated the effect of freezing and thawing on urine protein profiling using surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry. In that study, storing samples at (–70°C) and thawing at room temperature (up to four cycles) produced no evidence of protein degradation, although some protein peak loss was seen after five freeze–thaw cycles [36]. Our hypothesis was that a small number of freeze–thaw cycles would yield more reproducible measured VEGF levels than a greater number of cycles.

In the third part of the experiment, the relationship between different brands of polypropylene tubes used for sample storage and measured urinary VEGF levels was tested. Various brands of commercially available polypropylene tubes can differ in transparency, metal content, and contaminant levels. Endotoxin detection studies reveal that different brands of polypropylene tubes may not be equivalent for certain diagnostic tests [37]. We postulated that the differences between three types of polypropylene tubes would be minor and statistically insignificant in the context of measuring VEGF using a chemiluminescent immunoassay.

The final factor that was tested for its effect on measured VEGF level was the inclusion or exclusion of urinary sediment. Urinary sediment can contain erythrocytes, leukocytes, transitional cells, tubular cells, squamous epithelial cells and various types of casts and crystals [38]. It is not known whether any of these contain sources of VEGF or if they are capable of interfering with the immunoassay. To investigate this question, we separated the urinary sediment from the supernatant to see if the sediment influences measured VEGF levels.

Materials and methods

Cancer patients

Specimen collection

Human urine samples were collected from nine cancer patients undergoing radiation therapy at the National Cancer Institute. The patients were instructed to provide midstream urine specimens at the time of their scheduled visits to the radiation oncology clinic. All samples were obtained with informed consent in the context of an institutional review board-approved protocol at the National Cancer Institute.

Specimen processing

Each sample was immediately vortexed to mix the contents and divided into 10 aliquots. The aliquots were processed as follows:

1. Freeze-delay: Four 1-ml aliquots were transferred to 1.5 ml microcentrifuge tubes. The first was frozen immediately at –20°C, the second 1 hr post-collection, the third at 4 hrs, and the fourth at 24 hrs.
2. Tube type: Three 1-ml aliquots were placed in three types of polypropylene tubes – a 1.5 ml microcentrifuge tube (Eppendorf AG, Hamburg, Germany), and two types of 5 ml cryogenic vials, (Corning, Inc., Corning, NY), and (Greiner Bio-One International AG, Kremsmuenster, Austria). All samples were frozen immediately at –20°C.
3. Number of freeze–thaw cycles: Aliquots were placed in five 1.5-ml microcentrifuge tubes and frozen immediately. One aliquot was thawed once before measurement (one freeze–thaw cycle). The next aliquot was thawed at room temperature for 3 hrs, and then refrozen before rethawing for measurement (two freeze–thaw cycles). This was continued for each aliquot up to five freeze–thaw cycles.

Healthy volunteers

Midstream urine samples were also obtained from six healthy volunteers. Each urine specimen was mixed, then 10 ml placed in a conical centrifuge tube and centrifuged at 470 x *g* for 10 min at 4°C. Eight 1-ml aliquots were then taken from the supernatant and pipetted into microcentrifuge tubes. The remainder of the sample was vortexed to re-suspend the sediment in the final 2 ml of urine. Finally, a 1-ml aliquot was taken of this sediment resuspension. The sediment re-suspension and the first six samples of supernatant were frozen immediately. The next supernatant sample was frozen at 4 hrs and the last at 24 hrs, post-collection. All samples were frozen and stored at –20°C until analysis.

VEGF measurement

Each aliquot was thawed at room temperature for 3 hrs before measuring VEGF levels, in accordance with the procedure outlined by Kirk *et al*. in this issue. Urinary VEGF concentrations were determined using a commercial chemiluminescent immunoassay (QuantiGlo® system; R&D systems, Minneapolis, MN) according to the manufacturer's instructions. Each sample was run in triplicate by a single experienced technician who was blinded to the sample's preparation method.

Statistical methods

For each sample, the mean VEGF concentration and standard error were calculated from the triplicate assay data. Unpaired t-tests with unequal variances were used to compare individual samples processed in each of the ways described, and paired t-tests were used to compare over the set of samples.

Results

Cancer patients

Nine samples from cancer patients were analysed for urinary VEGF concentration. The average VEGF concentration for samples that were frozen immediately was 52.3 pg/ml (range 1.36–302 pg/ml).

Effect of freeze-delay time on measured urinary VEGF levels

In each of the nine samples, there was no significant difference between urinary VEGF levels in samples frozen at 0, 1 and 4 hrs post-collection at the 95% confidence level (Fig. 1, unpaired t-tests, two-tailed with unequal variances; Table 1, paired t-tests, two-tailed). There was a non-statistically significant trend toward increased urinary VEGF levels in samples left at room temperature for 24 hrs before freezing ($P = 0.16$, paired two-tailed t-test), as seven of the nine samples tested showed elevated VEGF concentrations.

Effect of increasing the number of freeze–thaw cycles on measured urinary VEGF levels

Measured VEGF levels were not statistically related to the number of freeze–thaw cycles between one and five (Fig. 2). Likewise, there was no statistically significant difference in VEGF levels between any two freeze–thaw cycles (Table 2).

Effect of tube composition on measured urinary VEGF levels

At the 95 % confidence level, there was no statistical difference in measured VEGF concentrations for three different tube types using paired t-tests (Fig. 3). Unpaired t-tests gave a statistically significant difference

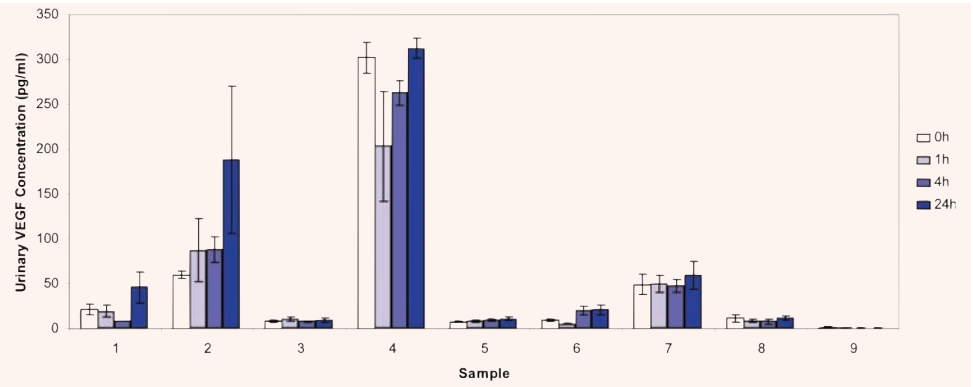

Fig. 1 Effect of freeze-delay time on urinary VEGF concentrations for nine cancer patients. Values presented are means ± SEM ($n = 3$).

Table 1 The statistical significance of delayed freezing

Time (hrs)	0	1	4	24
0	-	0.48	0.75	0.16
1	0.48	-	0.36	0.08
4	0.75	0.36	-	0.08
24	0.16	0.08	0.08	-

P-values: paired t-test two tailed

only for sample 2 and only when comparing the Eppendorf and Corning tubes ($P = 0.005$).

Healthy volunteers

Patients receiving radiation therapy represent a variety of cancer types and have received various chemotherapeutic agents, including nephrotoxic drugs. For this reason, we preferred to use specimens from healthy volunteers. Samples from six healthy volunteers were separated into sediment and supernatant and the VEGF concentrations were measured.

The presence of sediment increased VEGF levels in urine samples of healthy volunteers

In five of six samples, the sediment-containing sample gave a higher VEGF level than the sample containing only supernatant (Fig. 4), but the result was not statistically significant across all six samples ($P = 0.36$, paired two-tailed t-test). When compared individually, only sample 5 was found to have a statistically different level of VEGF between sediment and supernatant ($P = 0.012$, unpaired t-test with unequal variances, two-tailed).

Effect of freeze-delay time on sediment-free samples of healthy volunteers

To verify that the previous time-to-freeze data were not affected by the presence of sediment, the time-to-freeze experiment was repeated with sediment-free samples from healthy volunteers. As with the previous data, statistically significant changes in VEGF levels in freeze-delayed samples were not seen up to 4 hrs post-collection ($P = 0.25$, paired t-test, two-tailed, data not shown). Thus, with or without sediment, samples can be processed up to 4 hrs post-collection without significant alterations in VEGF levels.

Discussion

In this study, we evaluated the effects of tube type, time to freeze, number of freeze–thaw cycles and the presence of urinary sediment on measured levels of VEGF in human urine. Previous studies have examined the significance of urinary levels of VEGF at presentation and follow-up, but little was known about the way in which urine specimens must be handled to provide consistent results.

Our first hypothesis was that proteases in the urine degrade VEGF over time and that VEGF levels would decline over time when the urine was left at room temperature for extended periods before freezing for storage. We found no statistical evidence of this trend. In fact, VEGF levels tended to increase over time, although the effect was not statistically significant. Samples left on the bench top at room

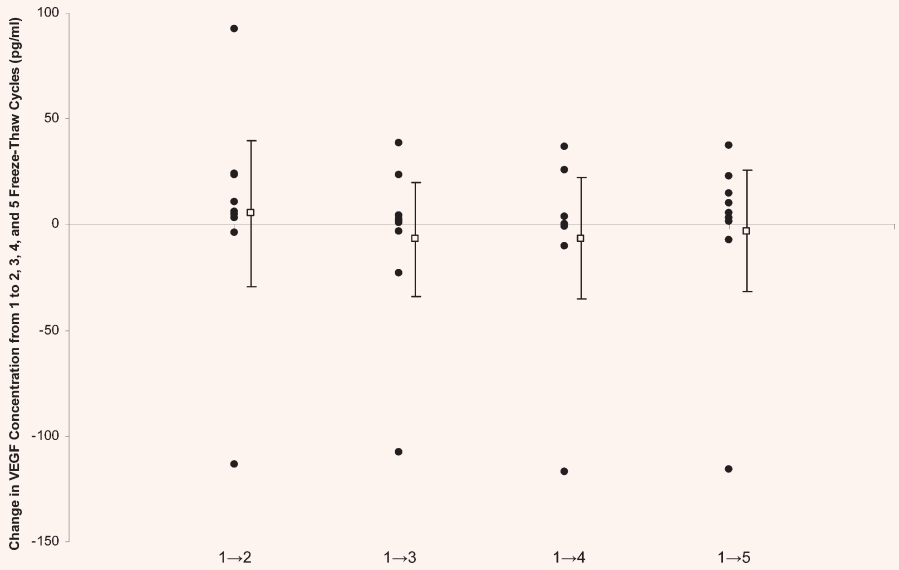

Fig. 2 The change in urinary VEGF concentration from 1 to 2, 3, 4, and 5 freeze–thaw cycles for nine cancer patients. (▭) Mean value (n = 3); (error bars) 95% confidence interval.

temperature for up to 4 hrs provided reproducible results; therefore we recommend freezing urine specimens within 4 hrs.

Multiple freeze–thaw cycles were not found to be significant in the context of measured VEGF levels. It should be noted that a zero freeze–thaw cycle was not tested, since it is impractical to run the VEGF assay on one fresh urine sample at a time. Our hypothesis was that a small number of freeze–thaw cycles would allow reproducible VEGF measurements, but that a greater number would not. This is consistent with results reported by Schaub *et al.*, in which the effects of freezing and thawing on urine protein profiling were evaluated. In that study, up to four cycles of freezing and thawing produced no evidence of protein degradation, although some protein peak loss was seen after five freeze–thaw cycles [36]. Unlike Schaub's results, a statistically significant decrease in protein concentration after five freeze–thaw cycles was not observed in the current study. Possible explanations for this difference include variations in freeze–thaw stabilities between different proteins and mass spectrometry's greater precision in protein quantification, which allows detection of smaller changes in protein concentration.

Next, we examined the effect of tube type on urinary VEGF levels. We postulated that the differences in three types of polypropylene tubes would be minor and that these differences would not affect VEGF levels measured using a chemiluminescent immunoassay. This was found to be the case for eight of nine samples, but one sample showed statistically significant results when comparing between two tube types. Given that 18 t-tests were calculated, the chance of at least one false positive at the 95% confidence level is approximately 60%. Thus, this result is not entirely unexpected, given the hypothesis that polypropylene tube type does not influence VEGF levels.

Table 2 The statistical significance of freeze–thaw cycles

Freeze–thaw cycles	1	2	3	4	5
1	-	0.77	0.63	0.66	0.84
2	0.77	-	0.15	0.11	0.37
3	0.63	0.15	-	0.91	0.33
4	0.66	0.11	0.91	-	0.22
5	0.84	0.37	0.33	0.22	-

P-values: paired t-test two tailed

Urinary sediment was found to have a higher measured VEGF concentration than supernatant in five of six samples, although the effect was only statistically significant for one sample. The sediment may contain a source of VEGF or the presence of

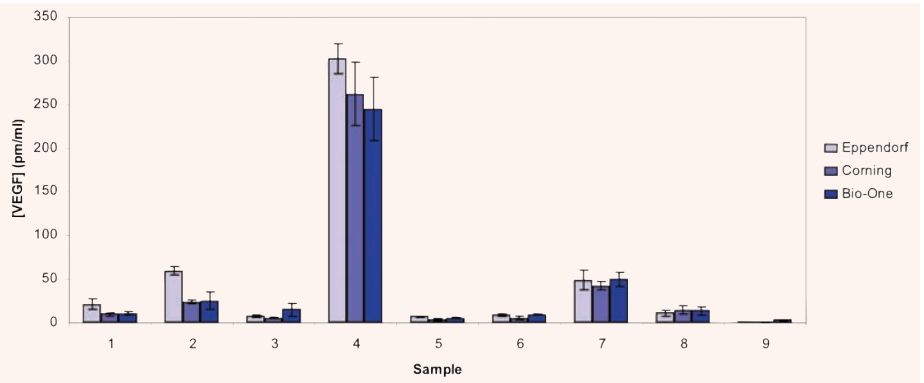

Fig. 3 Urinary VEGF concentration versus storage tube type for nine cancer patients. Values are expressed as means ($n = 3$) ± SEM.

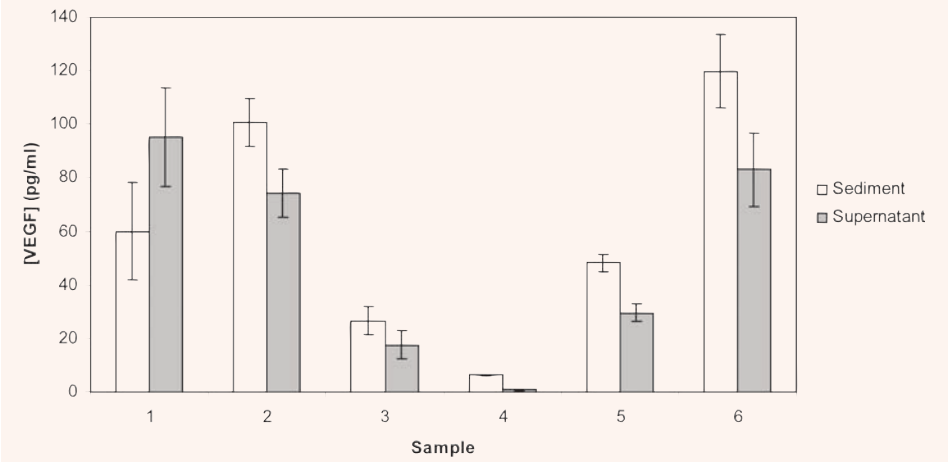

Fig. 4 Average VEGF concentration ($n = 3$) in sediment and supernatant for six healthy volunteers. (Error bars) standard errors.

sediment in the chemiluminescent kit may be falsely elevating the results. Studies to determine the cause of increased VEGF in urinary sediment are ongoing. We recommend centrifuging and removing the sediment from samples before freezing. In this study, centrifugation was performed at 470 x *g* for 10 min, but centrifugation with greater force has been employed by others [36, 39].

One caveat of this type of study is that the precision of the VEGF assay is critical to the statistical significance of the results. Large standard error values were seen for some of the samples run in triplicate. A more precise assay may have revealed differences between processing methods that were found to be statistically equivalent in this study.

In conclusion, the postulated effectors of VEGF concentration: freeze-delay (less than 4 hrs), number of freeze–thaw cycles (less than or equal to five), and tube type did not affect VEGF levels in a statistically significant way. However, these tests were dependent on the precision of the assay. The presence of sediment increased VEGF levels in five of six urine samples tested. Several postulated mechanisms exist for this increase, but none of them are accompanied by a theoretical reason why the sediment VEGF would have predictive value across a range of cancers since circulating tumour cells are not expected to traverse the glomeruli. Thus, our standard operating procedure is to remove sediment before sample storage and to store the sediment separately, resuspended in a standard volume of urine in the event that it becomes analytically useful. In addition, we recommend the use of samples within five freeze–thaw cycles.

Acknowledgements

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