Vascular endothelial growth factor (VEGF) is released from platelets during blood clotting: implications for measurement of circulating VEGF levels in clinical disease

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(Received 26 August/31 October 1997; accepted 5 November 1997)

1. Dysregulated vascular endothelial growth factor (VEGF) expression has been reported in several pathological states based upon evidence of elevated serum VEGF levels. Using two immunoassays for VEGF, this study determines normal plasma and serum VEGF ranges, determines which are more likely to reflect circulating VEGF levels and investigates a potential contribution of VEGF from platelets to VEGF levels detected in serum.

2. The presence of soluble VEGF receptor, sflt-1, at a molar excess of 7:1 significantly reduced measured VEGF levels in both assays. Serum VEGF levels were higher than plasma levels in children [(mean ± S.E.M.) 306.1 ± 39.4 versus 107.4 ± 24.9 pg/ml, \(P < 0.0001\)] and adults (249.4 ± 46.4 versus 76.1 ± 10.7 pg/ml, \(P < 0.0001\)). Serum VEGF increased with clotting time (\(P = 0.0005\) compared with 2 h samples); plasma VEGF levels were not affected by time between sampling and centrifugation.

3. Calcium-induced clotting of platelet-rich but not platelet-poor plasma induced VEGF release with a proportional response between platelet count and VEGF level and isolated platelets released significant quantities of VEGF upon incubation with thrombin. Reverse transcriptase-PCR studies confirmed that platelets express VEGF121 and VEGF165 mRNA.

4. These data suggest that plasma is the preferred medium to measure VEGF levels; a significant and highly variable platelet-mediated secretion of VEGF during the clotting process invalidates the use of serum as an indicator of circulating VEGF levels in disease states.

INTRODUCTION

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is a disulphide-linked homodimer of 34–42 kDa. It is an endothelial cell mitogen [1] which promotes angiogenesis and also has potent vascular permeability enhancing properties [2]. Alternate splicing of mRNA results in the generation of four protein species of 121, 165, 189 and 206 amino acids. On the basis of protein structure, particularly the pattern of conserved cysteines and sequence similarity, VEGF has been considered to be a member of the platelet-derived growth factor protein family [3].

VEGF mediates biological function by interaction with two specific tyrosine kinase receptors on endothelial cells: flt-1 (fms-like tyrosine kinase) [4] and KDR (kinase insert domain containing receptor) [5], also known as VEGFR1 and VEGFR2 respectively. The flt-1 gene also undergoes alternate splicing to produce both membrane-bound and soluble receptor variants. The soluble variant, sflt-1, has been shown to neutralize the effects of VEGF in vitro [6], although as yet there is no clear evidence to support a similar role in vivo.

Dysregulated VEGF expression has been implicated in a number of pathological situations, including tumour angiogenesis/metastasis [7], rheumatoid arthritis [8–10], diabetic retinopathy [11, 12], glomerular disease [13] and ovarian hyperstimulation syndrome [14]. Elevated serum VEGF levels have also been detected in women with pre-eclampsia [15] and in patients with a variety of cancers [16]. The ability to measure circulating VEGF protein levels is therefore clearly of importance to clinical researchers in a wide range of specialities.

A variety of immunoassays using combinations of polyclonal and monoclonal antibodies have been described for measurement of VEGF, predominantly in serum, by a number of authors [16–19]. It is unclear how these assays relate to each other in terms of sensitivity and specificity for different forms of VEGF and no comparison of different recombinant VEGF (rVEGF) standards has been undertaken. Importantly, there has been no validation of the use of serum VEGF as a suitable indicator of

Key words: immunoassays, platelets, vascular endothelial growth factor, vascular permeability factor.

Abbreviations: mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; RT, reverse transcriptase; VEGF, vascular endothelial growth factor.

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circulating VEGF levels. A recent study showing that stimulated platelets secrete VEGF [20] raises the possibility that VEGF detected in serum is an artefactual consequence of platelet activation during blood clotting and that only plasma values reflect the circulating level of VEGF.

The aims of this study were to (i) describe a soluble receptor-mediated capture assay for VEGF, compare its performance with that of a monoclonal-antibody (mAb)-mediated capture assay and investigate the potential for inhibition of VEGF signal by the presence of sflt-1 in both assays, (ii) determine the normal paediatric and adult VEGF ranges in plasma and serum using these two different assays, (iii) determine whether plasma or serum measurements are more likely to reflect circulating VEGF levels and (iv) to investigate a potential contribution from platelets to measured VEGF levels in serum.

**MATERIALS AND METHODS**

Recombinant (baculovirus-derived) VEGF165 and sflt-1 were kindly donated by Dr Don Ogilvie, Zeneca Pharmaceuticals (Alderley Edge, U.K.). VEGF121, VEGF ELISA kits, recombinant interleukin-1, 2, 3, 4, 6, 8 and 10, tumour necrosis factor-α and interferon-γ were all purchased from R&D Systems (Abingdon, U.K.). Peroxidase-conjugated donkey anti-rabbit and goat anti-mouse antibodies were purchased from Jackson ImmunoResearch (Luton, U.K.). Mouse monoclonal anti-VEGF, phorbol 12-myristate 13-acetate and thrombin were all purchased from Sigma Chemical Co (Poole, U.K.). Optiprep was purchased from Robins Scientific (Knowle, U.K.). Taq DNA polymerase, reverse transcriptase, RNase inhibitor, oligo(dT)12-18, 100 bp DNA ladder and dNTP solutions were purchased from Life Technologies (Paisley, U.K.).

**Production of polyclonal antibodies**

Male New Zealand White rabbits were immunized by subcutaneous injection of 50 μg of rVEGF; booster doses of 50 μg were given at 4 and 8 weeks. IgG isolation was performed by affinity purification by passage over a Hitrap column coated with rVEGF. Bound antibodies were eluted from the Hitrap–VEGF column with 0.1 mM glycine, pH 2.5, neutralized with solid Tris to pH 7.0 and concentrated to approximately 0.5 mg/ml. All animal care was in accordance with U.K. Home Office guidelines.

**Assay techniques**

- **flt-1 capture assay.** sflt-1 (1 μg/ml in 0.05 M carbonate buffer, pH 9.6, 100 μl/well) was adsorbed on to 96-well plates (MicroFLUOR W, Dynatech Laboratories U.S.A.) for a minimum of 16 h at 4°C. The plates were washed 10 times in wash buffer (0.1 M PBS, pH 7.2, 0.05% Tween 20) before and after being blocked with assay buffer (5% BSA in PBS, 150 μl/well) for 1 h on an agitator at room temperature. The wells were then filled with either samples, standards or controls (100 μl/well). Plasma or serum samples were added undiluted. The standard curve was generated using Zeneca rVEGF [rVEGF(Zen)] diluted in assay buffer and ranged from 19 pg/ml to 10 ng/ml in nine serial 2-fold steps. Background wells contained assay buffer alone. The culture supernatant from U937 cells (monocytic cell line, ECACC no. 85011440) stimulated with phorbol 12-myristate 13-acetate, 5 ng/ml for 22 h, acted as a positive control for ascertainment of inter- and intra-plate variability. Plates were incubated for 16 h at 4°C after which they were washed 10 times in wash buffer. Affinity-purified rabbit-anti-VEGF polyclonal antibody (dilution 1:500 in assay buffer, 100 μl/well) was then added for 4 h on an agitator at room temperature followed by a further 10 washes. The peroxidase-conjugated goat anti-rabbit IgG (heavy- and light-chain-specific) diluted 1:500 in assay buffer, 100μl/well, was then added and the plate was incubated at room temperature on an agitator for 2 h followed by a further 10 washes. The plate was then developed using the Amerlite buffer and tablet packs (Amersham International, U.K.) according to the manufacturer's instructions and read on an Amerlite enhanced luminescence microtitre plate reader (Berthold, U.K.). To assess the assay specificity, plasma from a healthy adult shown to have undetectable levels of VEGF and a 5% BSA solution were spiked with the following cytokines at a concentration of 5 ng/ml; interleukin-1, 2, 3, 4, 6, 8 and 10, tumour necrosis factor-α and interferon-γ.

**mAb-mediated capture assay.** This was performed according to the manufacturer's instructions (R&D Systems). Briefly, plasma, serum or cell culture supernatant samples and a rVEGF [rVEGF(R&D)] standard curve (31.2 pg/ml to 2 ng/ml) were added in assay diluent to precoated wells and incubated for 2 h at room temperature. After a standard washing step, anti-VEGF conjugate was added to each well and incubated for 2 h at room temperature. After a further washing step, substrate solution was added to each well for 20 or 25 min, after which stop solution was added and the plate read on a Molecular Devices ThermoMax plate reader at 450 nm. The culture supernatant from U937 cells as described above was also used to ascertain inter- and intra-plate variability with this assay. The assay has been shown to be specific for VEGF with no interference from a range of cytokines tested at 50 ng/ml.

As different rVEGF sources were being used to generate the standard curves in these assays [rVEGF(Zen)] with the flt-1 capture assay and rVEGF(R&D) with the mAb-mediated capture
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Determination of normal paediatric and adult VEGF ranges; sample collection and storage

Blood samples were collected from healthy adult volunteers (n = 34) and also from otherwise healthy children undergoing minor surgical procedures under general anaesthesia (n = 19). Paediatric samples were taken immediately after the induction of anaesthesia before the commencement of surgery. A separate venepuncture site was chosen rather than using the intravenous cannula to obtain samples to avoid any contamination by intravenous anaesthetic agents. Whole blood was collected into Becton and Dickinson (B&D) plain glass and EDTA (0.072 ml of 7.5% EDTA per 3 ml sample) blood tubes at room temperature, and centrifugation of samples and separation of serum and plasma was performed within 2 h of venepuncture. Serum and plasma samples were stored in multiple aliquots at −80°C before assaying using both assay systems. The samples were then measured in duplicate on both assay systems.

Investigation of a potential contribution from platelets to measured VEGF levels in serum

To investigate whether release of intra-cytoplasmically stored or membrane-bound VEGF during the clotting process contributes to VEGF levels detected in serum, 3 ml EDTA blood samples were taken from six adult volunteers. The samples were microfuged at 13 000 rpm for 10 min in order to obtain a cell pellet containing erythrocytes, leucocytes and platelets. The platelet-poor plasma was then removed and the cell pellet washed three times in PBS to remove any remaining plasma or plasma products. The pellet was then resuspended to a total volume of 3 ml in assay buffer and revolved on a rolling plate for 15 min at room temperature before respinning and assaying of the supernatant using the mAb-mediated capture assay.

To investigate whether platelets contribute to the release of VEGF during the clotting process, whole blood samples were taken from six adult volunteers by free flow into citrated blood tubes via an indwelling 19 G cannula (thus avoiding the activation of platelets which occurs when blood samples are drawn into a syringe via a narrow bore needle). Samples were centrifuged at 170 g for 10 min and the platelet-rich plasma removed and then divided into two portions. One portion was centrifuged again at 2700 g for 15 min to produce platelet-poor plasma [21]. Coulter counter analysis (Technicon H3) was performed on these plasma samples to quantify the platelet content and the degree of leucocyte contamination. One-millilitre samples of both platelet-rich and platelet-poor plasma were then placed in glass tubes and calcium chloride was added to a final concentration of 4 mM to induce clotting at room temperature. Unstimulated samples acted as controls. At time 0 (t0) and at 2 h, samples were centrifuged and the plasma/serum removed and frozen at −80°C before assaying using the mAb-mediated capture assay. For each individual, two parallel 2 ml whole blood samples collected in a plain glass tube were either centrifuged immediately or allowed to clot for 2 h before centrifugation and removal of the serum. In a similar experiment, to
confirm whether a platelet ‘dose-response’ was present, platelet-rich plasma was serially diluted with autologous platelet-poor plasma to achieve final platelet concentrations of between 4 and $150 \times 10^9/l$ and calcium chloride (4 mM final concentration) then similarly added to all samples.

In further experiments, after venepuncture of healthy adult volunteers ($n = 10$) using free flow from an indwelling 19 G cannula into citrated blood tubes, pure platelet populations were isolated using Optiprep™ density gradient centrifugation. The platelet count and purity were established using Coulter counter analysis. One-millilitre aliquots of isolated platelets were incubated with thrombin (10 units/ml) for 2 h at 37°C/5% CO2, with unstimulated platelets incubated under identical conditions acting as a control. Thrombin activity at this concentration was confirmed, the thrombin time being 18 s in a standard blood clotting assay. Two parallel whole blood samples collected from each individual into plain glass tubes were either centrifuged immediately or allowed to clot for 2 h at room temperature. At time 0 ($t_0$) and at 2 h, samples were centrifuged and the plasma/serum removed and frozen at −80°C before assaying using the mAb-mediated capture assay.

To investigate the expression of VEGF mRNA in platelets, pure platelet populations were isolated as above from three healthy adult volunteers, platelet count and purity being established using Coulter counter analysis. Total RNA was extracted using a modification of the guanidinium thiocyanate–phenol–chloroform method [22]. cDNA was synthesized from 5 μg of total RNA using superscript reverse transcriptase™ and oligo(dT)12–18 according to the manufacturer’s instructions. PCR amplification was performed using previously described primers known to amplify all reported VEGF splice variants [23] and CTAP-III[24], and β-actin primers designed to amplify cDNA sequence and not related processed pseudogene sequences [forward primer 5′-GCC GTC TIC CCC TCC ATC-3′ (nucleotides 125–143); reverse primer 5′-TAG CAA CGT ACA TGG CTG GGG-3′ (nucleotides 446–427, Genbank accession no. X00351), product size 322 bp]. PCR amplification was carried out in a Perkin–Elmer 480 DNA thermal cycler in 20 μl reactions overlaid with paraffin oil containing 1/25th (4%) of the volume of the cDNA reaction: 10 pmol of each primer, 0.75 mM of each dNTP, 10% DMSO, 16.6 mM (NH4)2SO4, 67 mM Tris/HCl pH 8.0, 85 μg/ml BSA and 0.5 units of Taq DNA polymerase. Thermal cycling conditions consisted of an initial denaturation step at 94°C for 3 min followed by 40 cycles for VEGF and 35 cycles for CTAP-III cDNA amplification at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min; and 30 cycles for β-actin cDNA amplification at 94°C for 1 min, 65°C for 1 min, 72°C for 1 min; and a final extension step at 72°C for 5 min. PCR products and a 100 bp DNA ladder (Life Technologies) were electrophoresed through 2% agarose gels, stained with ethidium bromide and visualized and photographed under UV illumination.

Statistical analysis

Paired and unpaired t-tests were used where data were determined to be normally distributed using the Kolmogorov-Smirnov test. The Wilcoxon matched pairs test was used for paired non-parametric data. Calculations were performed using GraphPad Prism™, GraphPad Software Inc, U.S.A.

RESULTS

Characteristics of the VEGF assays

The concentrations and orientation of the capture and detection proteins and antibodies for the flt-1 capture assay were determined and optimized in preliminary experiments. The resulant assay protocol is described in the Materials and Methods section and typical standard curves generated using rVEGF165 and rVEGF121 are shown in Fig. 1. The usual working range of the flt-1 capture assay was 78 pg/ml–10 ng/ml using VEGF165 for the standard curve; when VEGF121 was used, the standard curve showed saturation binding above 2.5 ng/ml. The inter-plate coefficient of variation, calculated from results of a total of 18 assays, was 11.49% with an intra-plate coefficient of variation, calculated using 10 measurements on a single plate, of 4.52%. The assay was found to be specific for VEGF, with cytokine/growth factor-spiked plasma and 5% BSA producing background values only (results not shown). The mAb-mediated capture assay is reported to detect both VEGF121 and VEGF165 and to be sensitive to 5–10 pg/ml, although the recommended standard curves have minimum standards of 31.2 pg/ml for use with plasma and serum samples and 15.6 pg/ml for culture supernatants. The inter-
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1. VEGF (pg/ml)

Fig. 1. Comparison of standard curves using both rVEGF165 (R&D) and rVEGF165 (Zen). Standard curves using rVEGF165 (R&D) and rVEGF165 (Zen) (31 pg/ml - 2 ng/ml) were measured using the mAb-mediated capture assay. ▲, rVEGF (Zen); ■, VEGF (R&D).

Platelet coefficient of variation, calculated from results of a total of 16 assays, was 16.1%.

That both recombinant VEGF standards behaved identically in the mAb-mediated capture assay is shown in Fig. 2, with standard curves for both proteins diluting out similarly. The rVEGF (Zen) used in theflt-1 capture assay, when read on the mAb-mediated capture assay, produced higher mean values than the rVEGF (R&D) (649 ± 22.4 pg/ml versus 523 ± 17.2 pg/ml; P = 0.0002, unpaired t-test). The equivalence ratio for rVEGF (Zen)/rVEGF (R&D) standards in this assay is 1.24:1. Furthermore, on measuring plasma samples containing significant quantities of VEGF in the two different assays, measured values were marginally higher in the mAb-mediated capture assay (r² = 0.646, P = 0.0029) (Fig. 3). The U937 supernatant positive control produced higher readings in the mAb-mediated capture assay (1502 ± 60.4 pg/ml) than in theflt-1 capture assay (980.2 ± 26.6 pg/ml) (P < 0.0001, unpaired t-test).

The effect of adding flt-1 to serum known to contain significant amounts of VEGF is shown in Fig. 4; measured VEGF values fell significantly with increasing amounts of added flt-1 in both the mAb-mediated and theflt-1 assays, showing 50% inhibition at a molar ratio of flt-1/VEGF of approximately 7:1. Therefore, both assays for VEGF are sensitive to the presence of soluble flt-1 and do not allow detection of VEGF in the form of a flt-1–VEGF complex.

Normal paediatric and adult plasma and serum VEGF ranges

Using both the mAb-mediated and flt-1 capture assays, paired plasma and serum samples were measured in a total of 19 children. The results are shown in Table 1. Serum VEGF levels were significantly higher than those in paired plasma samples [P < 0.0001, mAb-mediated capture assay; P = 0.0004, flt-1 receptor capture assay (paired t-tests)]. Table 2 shows normal adult plasma and serum ( clotting time 2 h) levels from a total of 34 adults. Twenty of these plasma samples were measured

![Graph](image1)

Fig. 3. Comparison of mAb-mediated capture and flt-1 assays. Plasma samples from normal adults were measured using both assays. Samples where circulating VEGF levels were above the background detection limits for both assays were compared using linear regression (r² = 0.646, P = 0.0029).

![Graph](image2)

Fig. 4. Effect of addition of flt-1 on measured VEGF levels in serum containing VEGF. rflt-1 [1.25–80 ng/ml (molar ratios 1:1–50:1)] was added to serum known to contain significant amounts of VEGF (750 pg/ml). After incubation for 2 h at room temperature, samples were measured using both the mAb-mediated capture assay and the flt-1 capture assay. VEGF levels are shown as a percentage of the value obtained in serum with no added flt-1. ▲, flt-1 capture assay; ■, mAb-mediated capture assay.

<table>
<thead>
<tr>
<th>VEGF (pg/ml)</th>
<th>mAb-mediated assay (n = 19)</th>
<th>flt-1 capture assay (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>107.4 (24.9)*</td>
<td>&lt;78#</td>
</tr>
<tr>
<td>Serum</td>
<td>396.1 (39.4)*</td>
<td>200.0 (26.4)†</td>
</tr>
</tbody>
</table>

*P < 0.0001 serum versus plasma levels (paired t-test); †P = 0.0004 serum versus plasma levels (paired t-test). All values below lower detection limit for assay.
using both assays; no significant difference was found in measured values between the two assays. Similar to the paediatric findings, serum levels were significantly higher than plasma levels ($P<0.0001$ unpaired $t$-test).

Do plasma or serum measurements reflect circulating VEGF levels?

The effect of clotting time on serum VEGF levels in 12 healthy adults is shown in Fig. 5. A significant rise in VEGF levels at 2 h compared with $t_0$ values ($P=0.0005$, Wilcoxon matched pairs test) was detected, with these elevated values persisting at 4 and 24 h. Two-hour serum VEGF levels were significantly higher in samples left to clot at $37^\circ$C [402.7 pg/ml (interquartile range 256.9–535.8)] compared with those left at room temperature [300.9 pg/ml (interquartile range 185.9–441.4)] ($P=0.03$, Wilcoxon matched pairs test). Plasma samples from 10 of these 12 adults left for similar time intervals before centrifugation and the removal of plasma showed no significant difference between $t_0$ and 2 h and later samples ($P=0.11$ paired $t$-test) (Fig. 6). VEGF in serum samples was stable after up to six repeat freeze–thaw cycles with no significant change in measured values (coefficient of variation 9.3%).

Contribution of platelets to VEGF levels in serum

Supernatant VEGF levels from total blood cell samples (erythrocytes, leucocytes and platelets) subjected to five freeze–thaw cycles ($n=6$ normal adults) were significantly increased (median 172.7 pg/ml, interquartile range 155.0–198.2 pg/ml) compared with levels in $t_0$ platelet-poor plasma (median 39.8 pg/ml, interquartile range 36.0–71.5 pg/ml; $P=0.03$, Wilcoxon matched pairs test). The addition of calcium to platelet-rich plasma (median platelet count $117 \times 10^9$/l, leucocyte contamination <0.1%) resulted in significantly elevated levels of VEGF release after clotting compared with platelet-poor plasma (median platelet count $9 \times 10^9$/l, leucocyte contamination <0.1%). VEGF levels in parallel control serum from whole blood samples are shown (Table 3). To provide further supportive evidence for the contribution of platelets to the increased VEGF levels detected in serum compared with plasma samples, VEGF levels in platelet-rich plasma made to clot by the addition of calcium chloride fell

![Graph](image1)

**Table 2. Normal values of VEGF in adult plasma and serum samples.** Values are means (SEM). Statistical significance: $^*P=0.32$, mAb versus fit-1 assay (20 paired plasma samples, paired $t$-test); $^{+}P<0.0001$ serum versus plasma (unpaired $t$-test).

<table>
<thead>
<tr>
<th>VEGF (pg/ml)</th>
<th>mAb-mediated assay ($n=34$)</th>
<th>fit-1 capture assay ($n=20$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>76.1 (10.7)$^{+}$</td>
<td>108.3 (11.5)$^{*}$</td>
</tr>
<tr>
<td>Serum</td>
<td>249.4 (46.4)$^{+}$</td>
<td></td>
</tr>
</tbody>
</table>

![Graph](image2)

**Table 3. VEGF levels after calcium chloride-induced clotting of platelet-poor and platelet-rich plasma samples ($n=6$ subjects).** Values are medians (interquartile ranges). Statistical significance: $^*P=0.03$, Wilcoxon matched pairs test; $^{+}P=0.03$, Wilcoxon matched pairs test. #All values below lower detection limit for assay.

<table>
<thead>
<tr>
<th>VEGF (pg/ml)</th>
<th>Platelet-poor plasma</th>
<th>Platelet-rich plasma</th>
<th>Normal serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_0$</td>
<td>34 ($31–56.5$)</td>
<td>$&lt;31$</td>
<td>48.5 ($31–81.5$)</td>
</tr>
<tr>
<td>2 h control</td>
<td>$&lt;31$</td>
<td>$&lt;31$</td>
<td>244 (203–382.5)</td>
</tr>
<tr>
<td>2 h CaCl$_2$ (4 mM)</td>
<td>$&lt;31$</td>
<td>$&lt;31$</td>
<td>114 (85–179.5)$^{*}$</td>
</tr>
</tbody>
</table>

![Graph](image3)
Platelet release of vascular endothelial growth factor

Platelet release of vascular endothelial growth factor (VEGF) was observed after the addition of calcium chloride to platelet-rich plasma. Platelet-rich plasma (platelet count \(150 \times 10^9/l\)) was serially diluted with autologous platelet-poor plasma (platelet count \(4 \times 10^9/l\)). Calcium chloride (final concentration \(4 \text{ mM}\)) was added to each sample which was then allowed to stand at room temperature for 2 h. After centrifugation, plasma VEGF levels were measured using the mAb-mediated capture assay.

As the platelet count of the platelet-rich plasma fell secondary to dilution with platelet-poor plasma (Fig. 7), isolated platelets (mean platelet count \(142 \times 10^9/l\), mean leucocyte contamination \(0.41\%\)) generated significant quantities of VEGF upon stimulation with thrombin compared with unstimulated control cells. VEGF levels in parallel serum from whole blood samples are shown (Table 4); a highly significant relationship was detected between 2 h serum VEGF levels and 2 h thrombin-induced platelet VEGF release (Fig. 8). Reverse transcriptase (RT)-PCR of mRNA from a pure population (leucocyte contamination <0.1%) of freshly isolated human platelets from three normal adults showed low but detectable PCR product corresponding to mRNA for the two soluble VEGF splice variants, VEGF121 and VEGF165 (Fig. 9). To eliminate contaminating leucocyte mRNA as the source of this detected PCR product, peripheral blood mononuclear cell (PBMC) cDNA was amplified by PCR using identical conditions to those used for the platelet cDNA amplification. PBMCs, which have previously been shown to express VEGF mRNA [23] and which were shown to be the predominant contaminating leucocyte in the platelet preparations by Coulter counter analysis, were isolated from whole blood (\(n=3\) normal adults) using standard ficoll density gradient centrifugation. Although the level of contamination of the platelet preparation by PBMCs was low (<0.1% by Coulter counter analysis), the relatively higher mRNA content of PBMCs

Table 4. VEGF levels after thrombin-induced clotting of purified platelet samples (\(n=10\) subjects). Values are medians (interquartile ranges). Statistical significance: *P = 0.0156, Wilcoxon matched pairs test. #All values below detection limit for assay.

<table>
<thead>
<tr>
<th>VEGF (pg/ml)</th>
<th>Platelets</th>
<th>Normal serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>to</td>
<td>&lt;31 #</td>
<td>32 (&lt;31–59.5)</td>
</tr>
<tr>
<td>2 h control</td>
<td>&lt;31 #</td>
<td>203.5 (154–349.5)</td>
</tr>
</tbody>
</table>
| 2 h thrombin (10 units/ml) | 116.5 (41–192.5)* | 607bp | 535bp | 403bp | 387bp | 322bp | Fig. 9. RT-PCR analysis of VEGF, CTAP-III and β-actin expression in human platelets. RT-PCR products were electrophoresed through 2% agarose gels and stained with ethidium bromide. Lanes 1 and 7 contain 1 μg of a 100 bp DNA ladder (Life Technologies); lanes 2, 3, 4 and 5 represent RT-PCR products after amplification of platelet cDNA from three different donors; lane 5 is a positive control (paediatric kidney) and lane 6 is a negative control (no cDNA template). Bands at 403, 535 and 607 bp correspond to VEGF121, VEGF165 and VEGF-α, respectively.
could theoretically produce a higher percentage contamination of the resultant cDNA preparation. To eliminate the possibility that PBMC-derived cDNA could be responsible for the observed PCR result, we tested PBMC-derived cDNA (Fig. 10A) and PBMC-derived cDNA diluted 1:20 and equivalent to 5% RNA contamination of the platelet preparation (Fig. 10B) under identical PCR conditions. As shown in Fig. 10B, no PCR product was detected, thus eliminating contaminating PBMCs as the source of the VEGF mRNA in Fig. 9.

DISCUSSION

The two assays described here have been shown to be specific for VEGF and to measure both soluble secreted VEGF forms, VEGF$_{121}$ and VEGF$_{165}$. The addition of flt-1 to serum containing a high concentration of VEGF reduced the detected VEGF level with a clear dose-response being observed; these findings show that neither the mAb-mediated capture assay nor the soluble receptor capture assay detect circulating VEGF–flt-1 complexes. It is likely, therefore, that the anti-VEGF antibody utilized in the mAb-mediated capture assay recognizes an epitope situated within or close to the flt-1 binding site so that the epitope is masked when VEGF is bound to flt-1. Both assays measured similar VEGF levels in normal human plasma and the measured difference in rVEGF at 500 pg/ml was small. We have previously tested other combinations of antibodies in an attempt to produce a more sensitive assay, in particular an affinity purified chicken polyclonal anti-VEGF (raised in-house) as capture and a monoclonal anti-VEGF antibody (Sigma V4758) as detection reagent, but we have been unable to improve significantly on the mAb-mediated capture assay described above (N.J.A. Webb, M.J. Bottomley, C.J. Watson and P.E.C. Brenchley, unpublished work).

We have reported for the first time the normal ranges of VEGF in children, and have shown normal range data in adult plasma samples. Previous publications have reported normal adult serum levels, although no comment was made about clotting times [19].

In attempting to establish normal ranges and to quantify VEGF in clinical samples by immunoassay, the first requirement is to confirm the validity of using either plasma or serum values and to determine whether either medium is sensitive to artefactual VEGF production or loss during or after sample collection. Our results in this study clearly demonstrate a significant difference in the normal values of VEGF for an individual depending on whether plasma or serum VEGF is measured, an issue which other published assay reports have not addressed. Serum values are invariably higher by a factor of approximately 6-fold with an apparent variable generation of VEGF signal between individuals over time for up to 4 h. This difference between serum and plasma VEGF levels has been noted with both the assays reported here and also with an in-house antibody capture assay using chicken anti-VEGF and monoclonal anti-VEGF reagents (results not shown). In a clinical situation, where blood samples are taken and left for variable times before processing, the contribution from the clotting process would effectively rule out the use of serum measurement. However, even if there was strict uniformity of clotting time for all samples, the large interpersonal variation in generation of VEGF in clotted samples makes interpretation of any observed difference between disease and control groups very difficult and may invalidate the results. This study is, therefore, the first to suggest that plasma is the preferred medium in which to measure in vivo circulating VEGF levels and to establish normal range data in plasma for both children and adults. Previous studies reporting elevated serum VEGF levels in various disease states compared with normal controls therefore need to be reviewed in light of these findings. There are an increasing number of reports of the use of rVEGF in human subjects as therapy for ischaemic injury [25, 26] which underlines the importance of accurately measuring circulating levels of the protein, taking into account the presence of sflt-1 which, if conjugated to VEGF, will prevent its detection with existing assay systems.

VEGF could be generated during blood clotting.

![Fig. 10. RT–PCR analysis of VEGF expression in human PBMCs.](image-url)
as a result of several mechanisms. Initially, we investigated the potential release of a preformed cytoplasmic or membrane-bound pool of VEGF from blood cells. Freeze–thawing of blood cell pellets generated a significant 4-fold increase in VEGF compared with \( t_0 \) plasma levels, suggesting that a potential reservoir of cytosolic or membrane-bound VEGF exists. When plasma was made to clot by the addition of calcium chloride, VEGF was released into the serum in significant amounts with platelet-rich but not with platelet-poor plasma, with a clear dose-response being seen. Purified platelets isolated from whole blood also released VEGF upon stimulation with thrombin. These data strongly support the role of platelets in this observed VEGF release after clotting.

The findings of previous studies investigating platelet VEGF mRNA expression are inconsistent; Katoh et al. [27] detected mRNA for VEGF121 only, whereas Charnock-Jones et al. [28] failed to detect mRNA using nested PCR primers, although no mRNA positive control lane for platelets was shown. More recently, Mohle et al. [20] identified VEGF secretion from a megakaryocyte cell line, ex-vivo-generated megakaryocytes and isolated platelets. In this study, we confirm platelets as a significant source of VEGF and in addition identify by RT–PCR mRNA for the two soluble secreted VEGF splice variants, VEGF121 and VEGF165. As the assay systems described detect both of the corresponding proteins, it is not possible to determine which is the predominantly expressed form.

It is possible that platelets are solely responsible for the VEGF released upon clotting of whole blood samples. VEGF levels in platelet-rich plasma and pure platelet samples were approximately 50% of those in parallel whole blood samples allowed to clot for an identical time period, although the median platelet count of 117 \( \times 10^9/\text{L} \) in the platelet-rich plasma and mean count of 142 \( \times 10^9/\text{L} \) in the pure platelet preparations reflects the incomplete recovery of platelets from whole blood.

An attractive hypothesis is that platelet activation during blood clotting induces VEGF secretion as part of the biological response to injury. As an immediate response, a pulse of VEGF released from platelets might alter local capillary permeability, allowing plasma proteins easy access to the site, provide a chemotactrant signal for monocytes, neutrophils [29] and endothelial cells [30] and stimulate endothelial cells into cell division and new capillary formation.

**ACKNOWLEDGMENTS**

This work was funded by grants from the North West Kidney Association, the Wellcome Trust and the Arthritis and Rheumatism Council, U.K. We would like to thank Dr Don Ogilvie of Zeneca Pharmaceuticals for the generous gift of recombinant VEGF and ft-1, and the Department of Haematology, Royal Manchester Children’s Hospital, for performing the Coulter counter analysis.

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