Vascular endothelial growth factor is bound in amniotic fluid and maternal serum

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Introduction

The vascular endothelial growth factor (VEGF) family comprises VEGF (Gospodarovicz et al., 1989; Keck et al., 1989; Leung et al., 1989), VEGF-B (Olofsson et al., 1996), VEGF-C (Joukov et al., 1996), VEGF-D (Orlandini et al., 1996; Yamada et al., 1997) and placenta growth factor (PIGF) (Maglione et al., 1991). Of these secreted factors, VEGF is the most potent direct stimulator of vascular endothelial cell growth. VEGF also induces vascular permeability (Keck et al., 1989) and its expression is enhanced by hypoxia (Ladoux and Frelin, 1993). VEGF is known to be expressed in the placenta (Sharkey et al., 1993; Vuorela et al., 1997) and in several fetal tissues (Kaipainen et al., 1993).

Two specific endothelial cell receptors, VEGF receptor-1 (VEGFR-1) or fms-like tyrosine kinase-1 (flt-1) (de Vries et al., 1992) and VEGFR-2 or kinase insert domain-containing receptor (KDR) (Terman et al., 1994), are known to bind VEGF, and a soluble form of VEGFR-1 has been identified (Kendall and Thomas, 1993; Kendall et al., 1996). VEGFR-1 also binds PIgF with high affinity (Park et al., 1994). Serum VEGF concentrations have earlier been reported to rise during the first trimester of pregnancy (Evans et al., 1998), but it is not known to what extent the VEGF receptors or other possible factors regulate the bio-availability of VEGF and PIgF during early or later pregnancy.

The presence of some, so far unknown, gestational factors regulating angiogenesis seems obvious considering the strong local neovascularization occurring in the placenta and the placental bed. The original aim of this study was to measure VEGF and PIgF in amniotic fluid and serum during pregnancy, and to identify possible factors regulating their activity.

Materials and methods

Subjects and sample collection

Pregnant subjects

After approval by the local ethics committee and informed consent, 40 healthy non-smoking women (median age 32 years, range 18–42 years) with uncomplicated pregnancies had venous blood samples drawn at gestational weeks 38–40, within the last 24 h before delivery, and on the third post-partum day (n = 21). Immediately after delivery umbilical cord venous (n = 24) and arterial (n = 13) samples were collected. Maternal venous blood samples of early pregnancy (weeks 10–13) were collected from 15 healthy women (median age 29 years, range 21–37 years). All pregnant subjects had uncomplicated singleton pregnancies and delivered healthy newborns.

Amniotic fluid

A pool of 50 random amniotic fluid samples from gestational weeks 15–17 was prepared from samples submitted for assessment of fetal chromosomal abnormalities and assay of α-fetoprotein (AFP). Eleven additional individual samples of amniotic fluid were collected during Caesarean section of healthy mothers at term. Samples were stored at –20°C until analysis.

Non-pregnant subjects

Single blood samples were collected from 22 healthy non-pregnant, non-medicated and non-smoking women (median age 29 years, range 17–39 years). As serum VEGF concentrations have been shown to correlate with serum progesterone concentrations (Evans et al., 1998),
all the samples were collected at the same phase of the menstrual cycle, i.e. the follicular phase.

Although no diurnal variation of urinary VEGF concentrations has been observed in gonadotropin-treated women (Robertson et al., 1995), all blood samples were collected between 8 and 9 a.m. Following separation, serum was frozen and stored at −20°C until analysis.

**Immunoaassays of VEGF and PlGF**

VEGF and PlGF were quantified by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions [Quantikine recombinant human (rh)VEGF and Quantikine rhPlGF, R&D Systems Europe Ltd, Abingdon, UK]. The detection limit of the assays was 16 ng/l. Recovery of rhVEGF and rhPlGF was tested by the ability of the ELISA to detect added rhVEGF (200 ng/l) or rhPlGF (100 ng/l) (proteins were provided as standard proteins in the ELISA) in various samples. The inter- and intra-assay coefficients of variation were 6.5 and 8.5% (Quantikine rhVEGF) and 6.3 and 7.9% (Quantikine rhPlGF). Recovery of added VEGF was also studied in serial dilutions of amniotic fluid and maternal serum samples from early and term pregnancy. According to the manufacturer, rhVEGF and rhPlGF ELISA show ∼20% and 5% cross-reactivity respectively, with the naturally occurring PlGF/VEGF heterodimer (DiSalvo et al., 1995). No cross-reactivity of either ELISA was observed with the novel growth factors VEGF-B (Olofsson et al., 1996) and VEGF-C (Joukov et al., 1996) when tested at concentrations 0.5–750 ng/l.

The possible influence of heparin on the ability of amniotic fluid to inhibit VEGF immunoreactivity in ELISA was studied by first incubating amniotic fluid with VEGF (see below) for 1 h at room temperature, after which heparin (0.05–5 mg/ml; Løvens Kemiske Fabrik, Ballerup, Denmark) was added to the samples and incubation was continued for 1 h at room temperature. VEGF concentrations were then measured by ELISA.

**Immunodiffusion**

The reactivity of the VEGF-binding factor with antibodies against α2-macroglobulin (anti-α2M, 7.2 g/l; DAKO, Denmark), pregnancy zone protein (anti-PZ, 5.8 g/l; DAKO) and pregnancy-associated plasma protein-A (anti-PAPP-A, 4.2 g/l; DAKO) was studied by immunodiffusion in 1% agarose gels. Samples of amniotic fluid and serum from pregnant women at term and from non-pregnant women were studied, either directly or following 1 h incubation with HCl for 15 min and neutralization with NaOH. Then [125I]VEGF was added to the samples and incubation was continued for 1 h at room temperature. VEGF concentrations were then measured by ELISA.
Table I. The concentrations of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) in serum and amniotic fluid. Values are expressed as mean ± SD

<table>
<thead>
<tr>
<th></th>
<th>VEGF (ng/l)</th>
<th>P value</th>
<th>PlGF (ng/l)</th>
<th>P value</th>
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<tbody>
<tr>
<td><strong>Serum</strong></td>
<td></td>
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<td>non-pregnant women (n = 22)</td>
<td>182 ± 147</td>
<td>&lt;0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16</td>
<td>&lt;0.001&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>early pregnancy (n = 15)</td>
<td>&lt;16</td>
<td>52 ± 23</td>
<td>&lt;0.05&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>term pregnancy (n = 40)</td>
<td>&lt;16 ng/l</td>
<td>439 ± 217</td>
<td>&lt;0.001&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>umbilical vein (n = 24)</td>
<td>502 ± 339</td>
<td>&lt;0.001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16</td>
<td></td>
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<tr>
<td>umbilical artery (n = 13)</td>
<td>421 ± 288</td>
<td>&lt;0.001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16</td>
<td></td>
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<tr>
<td>third post-partum day (n = 21)</td>
<td>137 ± 142</td>
<td>&lt;0.001&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21 ± 25</td>
<td>&lt;0.001&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td><strong>Amniotic fluid</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>early pregnancy (a pool of 50)</td>
<td>&lt;16</td>
<td>56</td>
<td>&lt;16</td>
<td></td>
</tr>
<tr>
<td>term pregnancy (n = 11)</td>
<td>&lt;16</td>
<td>30 ± 18</td>
<td>&lt;0.001&lt;sup&gt;d&lt;/sup&gt;</td>
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<sup>a,b,c</sup>Compared with pregnancy samples, maternal samples and term samples respectively.  
<sup>d</sup>Compared with term serum.

Results

Added VEGF was not recovered in serum samples of early and term pregnancy. However, full recovery of added VEGF was observed in post-partum maternal serum samples as well as in serum samples from umbilical artery and vein and from non-pregnant subjects. Added VEGF was not recovered in amniotic fluid from early or term pregnancy. However, full recovery of added PlGF was observed in all serum and amniotic fluid samples (Table I). Gestational age, birth weight of child or placental weight did not correlate with either serum VEGF or PlGF concentrations (data not shown).

Upon dilution of the maternal serum samples and the amniotic fluid samples, the inhibitory effect on added VEGF decreased, and 50% of added rhVEGF was recovered at approximately 1:500 dilution of amniotic fluid from early pregnancy and 1:1000 dilution of amniotic fluid from term. In maternal serum samples the inhibitory effect on VEGF was weaker than in amniotic fluid, and 50% recovery of added rhVEGF was observed at approximately 1:10 dilution of serum from early and at 1:30 dilution of serum from term pregnancy (Figure 1).

In gel filtration (S-300 HR, 85×1.5 cm column) [<sup>125</sup>I]VEGF eluted in fractions corresponding to an M<sub>r</sub> of 40 kDa (Figure 2A). When [<sup>125</sup>I]VEGF was pre-incubated with amniotic fluid from early or term pregnancy, the major radioactive peaks (78 and 73% of total radioactivity in the sample respectively) eluted in fractions corresponding to an M<sub>r</sub> of approximately 700 kDa. The remaining radioactivity eluted in fractions corresponding to an M<sub>r</sub> of about 40 kDa (Figure 2A). Following pre-incubation of [<sup>125</sup>I]VEGF with serum from early or term pregnancy, the major radioactive peaks (67 and 61% of total radioactivity in the sample respectively) shifted to fractions corresponding to molecular weights of 700 kDa or more and 400 kDa respectively (Figure 2B). When untreated maternal serum was separated on the same column, the corresponding fractions were found to inhibit recovery of added VEGF.

When [<sup>125</sup>I]VEGF was pre-incubated with serum from umbilical venous or arterial blood or serum of non-pregnant subjects, only minor radioactive peaks (17, 18 and 35% of total radioactivity respectively) eluted in the high molecular weight fractions (Figure 2C). The pattern was similar to that of [<sup>125</sup>I]VEGF alone suggesting polymerization of part of rhVEGF as a result of iodination.

When [<sup>125</sup>I]VEGF was incubated with amniotic fluid and then separated by gel filtration on a column (S-300 HR, 50×1 cm) equilibrated with 4 mol/l KSCN the elution profile was similar to that of [<sup>125</sup>I]VEGF alone. When [<sup>125</sup>I]VEGF incubated with amniotic fluid was separated in the same column using NaCl at concentrations of 0.5–4 mol/l in PBS, [<sup>125</sup>I]VEGF was not dissociated from the complex. Furthermore, the ability of amniotic fluid to shift the radioactive
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Figure 2. Sephacryl S-300 HR (85×1.5 cm) gel filtration analysis in tris-buffered saline showing binding of VEGF in amniotic fluid. A: [125I]VEGF alone or following pre-incubation with amniotic fluid (AF) of early and term pregnancy. B: [125I]VEGF following pre-incubation with serum (S) of early and term pregnancy. C: [125I]VEGF following pre-incubation with serum from umbilical vein or artery from non-pregnant women. Arrows at top of figure indicate molecular weight markers: 1, thyroglobulin 669 kDa; 2, ferritin 440 kDa; 3, human immunoglobulin G 168 kDa; 4, bovine serum albumin 67 kDa; 5, soybean trypsin inhibitor 20.1 kDa.

peak to high molecular weight fractions was retained after acidification of the complex to pH 2 and neutralization. However, when the [125I]VEGF complex was pre-incubated and separated at pH 2 without neutralization, no shift of the radioactive peak to the high molecular weight fractions was observed. Incubation with various concentrations of heparin before analysis in ELISA did not release VEGF from the complex in amniotic fluid.

In immunodiffusion with antibodies against α2M, PZ and PAPP-A no precipitation lines with amniotic fluid were observed, whether studied alone or after pre-incubation with [125I]VEGF. Serum of pregnant women at term showed precipitation lines with anti-α2M and anti-PZ, but not with anti-PAPP-A. Serum from non-pregnant women showed precipitation lines with anti-α2M, but not with anti-PAPP-A or anti-PZ. Autoradiography revealed bands corresponding to the precipitation lines for anti-α2M, whereas no bands were seen with PZ or PAPP-A (data not shown). By IFMA no α2M was observed in amniotic fluid.

In isoelectric focusing [125I]VEGF added to amniotic fluid, or to the VEGF-binding fractions of amniotic fluid or serum, displayed an isoelectric point of approximately 8. Addition of increasing amounts of non-radioactive rhVEGF caused dissociation of [125I]VEGF, which then focused in the pH range 4–5 (Figure 3).

Discussion

VEGF is a potent stimulator of vasculo- and angiogenesis. The extensive fetal and placental tissue growth of normal pregnancy is characterized by a strong local demand for vascular expansion, but so far little is known about factors regulating VEGF in human pregnancy.

The absence of detectable VEGF immunoreactivity in maternal serum in early and term pregnancy, followed by a post-partum rise in serum VEGF concentrations, suggested the presence of a pregnancy associated factor suppressing VEGF immunoreactivity in ELISA. Measurement of the recovery of VEGF added to serum showed the presence of a factor which probably bound VEGF. This activity was also detected in amniotic fluid at much higher concentrations. Gel filtration of [125I]VEGF added to pregnancy serum and amniotic fluid showed that this binding activity occurred in the high molecular weight fractions.
In early pregnancy, the VEGF-binding capacity was about 50 times higher in amniotic fluid than in maternal serum. The binding capacity in term amniotic fluid was two-fold that of early amniotic fluid. The origin and source, as well as the exact nature of the binding compound need further studies, but from the above data it can be concluded that the binding activity is strongly associated with gestation and dependent on gestational age. The high concentrations of the binding factor in amniotic fluid suggest that it originates from the amniotic fluid compartment. Interestingly, in gel filtration the complex formed in early amniotic fluid showed two distinct components, whereas term amniotic fluid contained only a single high molecular weight complex corresponding to the later eluting compound in early amniotic fluid.

The identity of the VEGF-binding compound is not yet known, but well known proteins of the corresponding molecular weight can be excluded. α2M, which is a major protease inhibitor in serum, has been shown to bind VEGF irreversibly (Soker et al., 1993), and in the immunodiffusion studies some radioactivity was found to be associated with α2M, but this could not be detected in amniotic fluid. Assays of α2M by IFMA confirmed that it is not detected in amniotic fluid (Bhat and Pattabiraman, 1980). Heparin did not affect VEGF binding in amniotic fluid or serum of pregnant women, whereas it has been reported to inhibit its binding by α2M (Soker et al., 1993). These results indicate that α2M is not responsible for the VEGF binding activity in amniotic fluid. However, on the basis of our immunodiffusion results, α2M binds some VEGF in serum of non-pregnant subjects, and it probably contributes to a minor part of the binding activity in maternal serum.

The serum concentrations of two high molecular weight proteins, PZ and PAPP-A have been shown to increase with advancing pregnancy, reaching peak at term, and declining during the post-partum days (von Schoultz, 1974; Lin et al., 1976). PAPP-A is also present in amniotic fluid (Bischof et al., 1992) during the post-partum days (von Schoultz, 1974; Lin et al., 1976). The results of this study suggest that the VEGF-binding activity of umbilical cord and post-partum maternal samples is low, and that most of the VEGF is unbound.

In conclusion, a putative heterodimeric high molecular weight VEGF-binding protein is present in maternal serum and amniotic fluid. Its concentrations are dependent on gestational age, it disappears after delivery, and it is not clearly detectable in umbilical blood. Because it does not bind PlGF, it does not appear to be the presently known soluble VEGF receptor. Further studies are needed to identify this VEGF-binding compound and to elucidate its potential role in the regulation of vasculogenesis of human pregnancy.

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References
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