Localization and Control of Expression of VEGF-A and the VEGFR-2 Receptor in Fetal Sheep Intestines

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ABSTRACT: We studied expression of vascular endothelial growth factor A (VEGF-A) and its main receptor, VEGFR-2, in the small intestine from five groups of fetal sheep (each $n = 5$): 1) preterm controls, 2) term controls, 3) preterm animals where the fetus was infused with cortisol, or 4) saline, and 5) term animals where adrenalectomy had been performed preterm. The main transcript expressed in fetal small intestine was VEGF-A₁₆₅. Comparing term with preterm animals, there were significantly higher levels of expression of VEGF-A protein ($p = 0.005$). Levels of VEGF-A protein expression were lower in term adrenalectomized animals ($p = 0.01$) and were higher in preterm animals infused with cortisol ($p = 0.01$), compared with their respective control groups. Immunohistochemistry demonstrated strongest expression of VEGF-A protein in the epithelial cells and lamina propria of the villi. Intestinal expression of mRNA encoding the VEGFR-2 receptor did not significantly vary with gestational age. *In situ* hybridization localized VEGFR-2 to the lamina propria of the villous core and receptor autoradiography using ¹²⁵I VEGF-A demonstrated binding in the same site. These data show that intestinal VEGF-A is up-regulated with advancing gestation in a glucocorticoid-dependent manner—novel findings consistent with a role for VEGF-A stimulated angiogenesis in preparing the fetal gut for birth. (*Pediatr Res* 63: 143–148, 2008)

Impaired intestinal function associated with preterm birth is a major source of morbidity and mortality in neonatal intensive care. Intestinal dysfunction is manifested by feeding intolerance, poor growth, malabsorption, and, in the most severe cases, necrotizing enterocolitis (NEC). The prevalence and severity of intestinal dysfunction varies inversely with the gestational age at birth (1). This inverse association suggests that changes occur in the fetal intestines with advancing gestational age in preparation for enteral feeding following birth. In many tissues, prepartum maturation in preparation for extrauterine life is dependent on an increase in glucocorticoid output by the fetal adrenal gland. In the gut, glucocorticoids induce a range of structural and functional changes that activate digestive function postnatally [see review (2)]. In sheep, these changes include increased enterocyte proliferation, decreased wall thickness, and induction of digestive enzyme activities (2).

Angiogenesis is a complex process where new capillaries are formed from preexisting vessels. Intestinal perfusion in neonatal life is essential for the transfer of absorbed nutrients following the commencement of enteral feeding, but little is known about the control of angiogenesis in the fetal intestine during the prepartum period of tissue maturation. One key growth factor involved in physiologic and pathologic angiogenesis is vascular endothelial growth factor-A (VEGF-A) (3). We hypothesized that 1) VEGF-A and its main functional receptor, VEGFR-2, are expressed in the fetal intestines; 2) one or both of these proteins are up-regulated in association with advancing gestational age; and 3) these maturational changes are mediated by glucocorticoids.

METHODS

Animal experimental procedures. Twenty-five Welsh Mountain sheep fetuses of known gestational age were used in this study. All the ewes were kept in pens individually and maintained on 200 g/d concentrates with free access to hay, water, and a saltlick block. Food, but not water, was withheld for 18–24 h before surgery. All surgical and experimental procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 under an appropriate Home Office license and with approval from the local ethical review process. The animal methods have been described in detail elsewhere (4). In brief, a laparotomy was performed under halothane anesthesia (1.5% in O₂-N₂O) between 115 and 118 d gestation (term 145 d) and, through a midline incision in the ewe, the uterus was partially exteriorized. One of two surgical procedures was carried out using the surgical methods previously described (5). In 10 animals, catheters were inserted into the caudal aorta and vena cava of the fetus *via* the tarsal vessels. This part of the study aimed to determine the effect of a precocious rise in fetal cortisol. This was achieved by comparing the effect of a 5-d period of cortisol infusion ($n = 5$, 2–3 mg/kg/d in 3 mL 0.9% wt/vol saline; EF-Cortelan; Glaxo Ltd., Greenford, Middlesex, UK) with a 5-d period of saline infusion ($n = 5$, 3 mL/d, 0.9% wt/vol), before tissue collection at 130 ± 1 d of gestation. This dose was chosen as it has previously been shown to mimic the prepartum rise in cortisol that occurs physiologically in the fetal lamb (6). Blood samples of 2 mL were taken daily throughout the experimental period from all the catheterized fetuses to monitor fetal well-being using blood pH, Po₂, and glucose levels (ABL5 Radiometer, Copenhagen, Denmark, and YSI glucose analyzer, YSI Inc., Yellow Springs, OH). In a further five cases, both fetal adrenals were removed at the time of the laparotomy. This part of the study aimed to determine the effect of preventing the prepartum rise in fetal cortisol, which normally occurs in late gestation. These animals were compared with five nonoperated, gestational age-matched controls ($n = 5$). All subsequent procedures were performed when the animals were conscious with an interval of at least 5 d following surgery. Tissue collection was performed near to term, *i.e.* 142–145 d gestation. We have previously shown that sham-operated and unoperated fetal sheep are comparable (7). Finally, a further five unoperated animals were delivered preterm (127 d gestational age) and were

Received July 5, 2007; accepted September 28, 2007.

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Supported by the Beebe Fund of Cambridge University and BBSRC.

Abbreviations: NEC, necrotizing enterocolitis; PIGF, placenta growth factor; RT-PCR, real-time PCR; SSC, sodium chloride/sodium citrate solution

compared with unoperated animals delivered at term to assess changes with advancing gestational age.

Fetuses were delivered for tissue collection by cesarean section under general anesthesia (20 mg/kg sodium pentobarbitone i.v.). At delivery, a 10-mL blood sample was taken from the umbilical artery, and tissues were collected from the animals after the administration of a lethal dose of barbiturate (200 mg/kg sodium pentobarbitone). Samples of fetal gastrointestinal tract were collected midway between the pyloric sphincter and ileo-caecal junction (jejunum) and at 20 cm proximal to the ileo-caecal junction (ileum). Tissues were either fixed in 4% paraformaldehyde or frozen in liquid nitrogen and stored at -80°C until analysis.

Cortisol assay. Fetal plasma cortisol concentrations were measured as described previously (8). After ethanol extraction, samples were analyzed by RIA with [^3H]cortisol as tracer. The lower limit of detection of the assay was 1.0–1.5 ng/mL. The intra- and interassay coefficients of variation were 5.3% and 13.0%, respectively.

Real-time PCR (RT-PCR). Total RNA was extracted from intestinal samples using a commercial kit (RNAqueous-4PCR, Ambion, Huntingdon, Cambridgeshire, UK). cDNA was synthesized using SuperScript III Rnase H⁻ reverse transcriptase (Invitrogen, Paisley, UK) from 2 μg of total RNA with an oligo dT₁₅ primer (Promega, Southampton, UK). To determine the VEGF-A splice variants present, RT-PCR using primers common to the different variants was used as previously described (9). Real-time quantitative PCR analysis was performed with an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA). The primers and fluorescently labeled probes for VEGF-A₁₆₅ and VEGFR-2 were synthesized by MWG-Biotech Ltd. (Milton Keynes, UK). VEGF-A₁₆₅-specific primers were based on the sequence described by Redmer *et al.* (9). Forward and reverse primers were 5' GCT CTC TTG GGT GCA TTG GA 3' and 5' GCC TGG GAC CAC TTG GC 3', respectively, and the FAM (6-carboxyfluorescein) labeled probe was 5' CCT TGC CTT GCT GCT CTA CCT TCA CCA 3'. The primers and probe for VEGFR-2 were developed from the sequence described by Scholz and Segar, 2000 Genbank Accession No. AF233076, using Primer Express software (Applied Biosystems, Foster City, CA). Forward and reverse primers were 5' GGC ATC ACG GAA GTG TAT CCA 3' and 5' CGT TCT TTT CCG ACA AGA GGA 3', respectively, and the FAM labeled probe was 5' AGG GAC CTG GCA GCG CGA AAC 3'. 18S ribosomal RNA was used as endogenous control and each sample was run in triplicate. Relative quantification of VEGF-A and VEGFR-2 mRNA to 18S ribosomal RNA was calculated by the comparative cycle threshold method.

Riboprobe synthesis. A riboprobe for VEGFR-2 was synthesized using a plasmid, which contained a cloned fragment of ovine VEGFR-2 cDNA. This was generated by the amplification of jejunum cDNA using the VEGFR-2 forward primer described above and the reverse primer 5'-ATA TTT TCC TTA GGT GCT TCT CCG-3'. The PCR reaction was heated for 1 min at 95°C and the amplification was carried out for 35 cycles (30 s at 95°C , 30 s at 62°C , and 30 s at 72°C), followed by a final extension period of 3 min at 72°C . The identity of the product (258 bp) was confirmed by cloning and sequencing in pGEM-T Easy Vector (Promega). To generate the riboprobe, plasmid DNA was linearized with *NcoI* or *SpeI* before *in vitro* transcription. Anti-sense and sense RNA probes were transcribed with ^{32}P -UTP (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK; 92.5 TBq/mMol), using the MAXIscript *in vitro* transcription kit (Ambion). To remove the plasmid template the probes were incubated with DNase at 37°C for 10 min, phenol extracted and ethanol precipitated.

In situ hybridization. Formalin fixed and paraffin embedded sections were dewaxed and pretreated with 0.2 N hydrochloric acid (HCl) for 20 min and proteinase K (2 $\mu\text{g}/\text{mL}$) (Sigma Chemical Co.) for 20 min at 37°C . Slides were then immersed in a solution containing 100 mM triethanolamine (pH 8) and 0.25% acetic anhydride twice for 5 min. Slides were washed in $2\times$ SSC for 5 min, dehydrated in ethanol serials, and dried. The probes were diluted to 30,000–40,000 cpm/ μL in hybridization buffer and denatured by boiling for 3 min. The hybridization buffer contained 0.3 M sodium chloride (NaCl), 10 mM Tris-HCl pH 6.8, 10 mM sodium phosphate pH 6.8, 5 mM EDTA, pH 8.0, 50% (vol/vol) deionized formamide, $1\times$ Denhardt's solution [0.02% (wt/vol) each of BSA, Ficoll, and polyvinyl pyrrolidone], 10% (wt/vol) dextran sulfate, 50 mM DTT (DTT), and yeast tRNA 1 mg/mL. The sections were incubated in a sealed container humidified with 50% formamide and 0.3 M NaCl for 18 h at 55°C .

The slides were washed in $5\times$ SSC at 50°C for 15 min twice, followed by $2\times$ SSC, 50% formamide at 65°C for 30 min. After washing four times for 5 min in $2\times$ SSC at 37°C , slides were incubated with RNase A (Sigma Chemical Co.) at a final concentration of 20 $\mu\text{g}/\text{mL}$ in $1\times$ wash solution containing 400 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 5 mM EDTA. The RNase A was removed by washing slides in $2\times$ SSC, 50% formamide at 65°C for 30 min. Slides were then washed in $2\times$ SSC and $0.2\times$ SSC at 37°C , 15 min each. Sections were dehydrated through 30%, 60%, 80%, and 95%

ethanol containing 0.3 M ammonium acetate and then two final washes in 100% ethanol. Slides were coated with autoradiography emulsion (LM-1 emulsion; Amersham International plc, Little Chalfont, Buckinghamshire, UK) and exposed for 6 wk at 4°C . Sections were developed (D19 developer; Kodak, Rochester, NY), fixed (Ilford Rapid Fixer), stained by hematoxylin, mounted, and photographed.

Immunocytochemistry. Localization of VEGF-like immunoreactivity was carried out as previously described (10) using a polyclonal anti-VEGF antibody (1:200, A-20; Santa Cruz Biotechnology, Santa Cruz, CA) on formaldehyde-fixed, wax-embedded material. The secondary antibody was goat anti-rabbit (1:300; DAKO, Ely, UK). Control sections were incubated with rabbit immunoglobulin fraction (DAKO) in place of the primary antibody. Slides were dehydrated, counterstained with hematoxylin, and mounted with DPX.

Quantitative microscopy. To quantify villous capillaries, immunohistochemistry was performed as described above using an antibody specific to von Willebrand protein. Primary antibody (DAKO) was added at a 1 in 1000 dilution (5.7 $\mu\text{g}/\text{mL}$). Slides were then incubated overnight at 4°C . Sections were washed and secondary antibody rabbit IgG-biotin (DAKO) was added at a 1 in 200 dilution. To quantify staining, images of the villi and crypts were captured and staining assessed using the Olympus Computer Assisted Stereological Toolbox (CAST Version 2.1.4., Olympus Danmark A/S 1999, Ballerup, Denmark). Between 30% and 60% of the area occupied by the crypts and villi was analyzed using meander sampling to record a minimum of 180 positively stained cells. Volume fraction analysis was then performed and brown-stained cells were expressed as a ratio to unstained cells. Assessment of volume fraction was performed blind to the gestational age of the animals.

Receptor autoradiography. Autoradiography was performed on sections of unfixed frozen tissue using ^{125}I -VEGF using the method of Shweiki *et al.* (11). Briefly, sections were preincubated in phosphate buffer 50 mM pH 6.5 containing 0.2% wt/vol gelatine and 1 mg/mL heparin for 1 h at room temperature. ^{125}I -labeled VEGF-A (Amersham Biosciences, 37–92.5 TBq/mMol) was added in the same buffer to a final concentration of 100 pM. To confirm specificity of binding and to identify the VEGF receptor type, slides were incubated with the addition of excess unlabeled VEGF or placental growth factor (PIGF) (final concentration 2.5 nM). All slides were incubated for 1 h at room temperature after which the sections were washed in ice-cold PBS, fixed in phosphate solution containing 2.5% vol/vol glutaraldehyde, and washed again in phosphate solution for 10 min, then ddH₂O for 1 min. Following air drying, the slides were coated with a thin film of autoradiographic emulsion (LM1 emulsion, Amersham International). Slides were stored in a desiccator at 4°C and then developed after 48 h. Once developed and counterstained with hematoxylin, sections were photographed under both bright- and dark-field illumination using a Leica (Wetzlar, Germany) DMR/B microscope.

Western blotting. Tissue was first ground to a powder in liquid nitrogen and then homogenized in ice-cold RIPA buffer (0.15 M NaCl, 5% Nonidet-40, 3% sodium deoxycholate, 0.5% SDS, 0.3 M Tris pH 8.0) containing complete mini protease inhibitor cocktail (Roche Diagnostics Ltd., East Sussex, UK). Tissue homogenates were centrifuged in 50-mL tubes at 3000 rpm for 10 min at 4°C to pellet cell debris. Supernatants were then centrifuged again at 14,000 rpm for 20 min at 4°C and the protein-containing supernatant removed. Protein concentration was determined using the bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL). Equal amounts of protein (20 μg per lane) were separated by electrophoresis on 10% polyacrylamide SDS gels. After electrophoresis, proteins were transferred to a polyvinylidene fluoride Immobilon-P membrane (Millipore, Bedford, MA), blocked with 5% skimmed milk in PBS-T for 30 min, and subsequently probed with an antibody to VEGF-A (1:500, VEGF-A A20; Santa Cruz Biotechnology) on a shaker at 4°C overnight or 1.5 h at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2000, DAKO) was used. To detect antibody binding, a chemiluminescent Western blotting detection system was used (Amersham Biosciences). Membranes were stripped with a buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β -mercaptoethanol at 50°C for 30 min. To correct for loading variations the membrane was reprobed with a rabbit polyclonal antibody to cyclophilin A (1:3000; Abcam, Cambridge, UK). The secondary antibody was goat anti-rabbit (1:4000, DAKO). The relative intensity of protein signals was normalized to the corresponding cyclophilin A density and quantified by densitometric analysis using public-domain software (Image J, National Institutes of Health).

Statistics. Groups were compared using unpaired *t* test using the Stata software package (Statacorp, College Station, TX). Statistical significance was assumed at $p < 0.05$ and all tests were two-sided. In addition, graphical presentation of Taqman quantitative RT-PCR was presented using estimated fold changes in expression (with 95% confidence intervals).

RESULTS

Mean cord blood cortisol levels at the time of delivery (measured in ng/mL) were compared among the different groups. Among uninstrumented animals, levels were lower in those preterm compared with term [mean (SEM): 16.1 (2.8) *versus* 91.8 (54.8), both $n = 5$, $p = 0.02$]. Among instrumented animals delivered preterm, levels were higher among those infused with cortisol compared with saline [mean (SEM): 91.2 (20.9) *versus* 14.1 (3.2), $n = 5$ and $n = 4$, respectively, $p = 0.01$]. Among animals delivered at term, levels were lower among those where adrenalectomy had been performed compared with unoperated animals [7.1 (0.6) *versus* 91.8 (54.8), both $n = 5$, $p = 0.009$].

Expression of splice variants of VEGF-A was studied using nested PCR with primers that were common to the main splice variants. This demonstrated two main bands, one of approximately 565 bases, consistent with VEGF-A₁₆₅ and one of approximately 433 bases, consistent with VEGF-A₁₂₁ (Fig. 1A). The band corresponding to VEGF-A₁₆₅ was consistently stronger. The expression of VEGF-A₁₆₅ was then studied using quantitative RT-PCR using specific primers and probes and expressed relative to 18S. This demonstrated a trend toward higher levels of expression in jejunum at term compared with preterm controls, but this did not reach statistical significance ($p = 0.1$, Fig. 1B).

Expression of VEGF-A protein was studied by Western blot using protein extracted from fetal sheep jejunum. This demonstrated a main band of 63 kDa. The antibody used was against mouse VEGF-A. We probed mouse kidney and found that the main band was also 63 kDa (data not shown). Moreover, preincubation of the antibody with the peptide used to raise the antibody abolished the band in both species, confirming specificity (data not shown). When the level of

Figure 1. Analysis of expression of VEGF-A. (A) Analysis of VEGF-A splice variants using RT-PCR using mRNA extracted from fetal sheep jejunum. Primers described in Methods. RNA from two preterm animals and two animals at term. Two signals seen were of interpolated size consistent with product of 565 bases (VEGF-A₁₆₅) and 433 bases (VEGF-A₁₂₁). (B) Comparison of levels of VEGF-A mRNA using Taqman analysis and quantifying fold change of VEGF-A relative to 18S ribosomal RNA using the comparative cycle threshold method. Points are means and bars are 95% confidence intervals and are expressed as the fold change referent to the preterm group. $*p = 0.1$. (C) Western blot for antibody specific to VEGF-A. (D) Quantitation of signal for VEGF-A protein relative to cyclophilin protein. Columns are means and bars are SEM. $**p = 0.005$.

Figure 2. Effects of manipulation of fetal cortisol levels on expression of VEGF-A protein in fetal sheep jejunum. Western blots for antibody specific to VEGF-A comparing (A) preterm animals infused with saline or cortisol, and (C) term animals that underwent adrenalectomy or a sham operation. Quantitation of signal for VEGF-A relative to cyclophilin, comparing (B) preterm animals infused with saline or control, and (D) term animals that were sham operated or had adrenalectomy performed. Columns are means, bars are SEM. $*p = 0.02$.

expression of VEGF-A protein was quantified densitometrically as a ratio to the housekeeping gene, cyclophilin, VEGF-A protein expression in jejunum was higher in term than preterm fetuses (Fig. 1, C and D). Preterm, VEGF-A protein expression in jejunum was higher in cortisol than saline-infused fetuses (Fig. 2, A and B). Term, VEGF-A protein expression in jejunum was lower in adrenalectomized than control fetuses (Fig. 2, C and D). Expression of VEGF-A protein was localized using immunohistochemistry. VEGF-A was expressed in the muscularis, the crypts, the epithelial cells, and lamina propria in both jejunum and ileum (Fig. 3). Binding was particularly marked in the lamina propria and

Figure 3. Immunohistochemistry using antibody specific for VEGF-A in fetal sheep small intestines. (A) Low power, jejunum. (B) Low power, jejunum negative control where antibody for VEGF-A replaced with rabbit IgG. (C) Low power, ileum. (D) High power, villous in jejunum. (E) High power, submucosa and muscularis in jejunum. Bar = 100 μ m.

Figure 4. *In situ* hybridization using riboprobe against VEGFR-2 in fetal sheep jejunum. (A) Dark field of low power using antisense probe. (B) Low power using sense probe (control). (C) High power of villous core of hematoxylin and eosin stained section. Bar = 100 μ m.

epithelial cells of the villi (Fig. 3C). Specificity of binding was confirmed by replacing the antibody for VEGF-A with non-immune rabbit IgG (Fig. 3B).

Reverse transcriptase-PCR using primers for the VEGFR-2 gene demonstrated a product of expected size (258 bp). The product was sequenced and the identity confirmed as VEGFR-2, and the product shared 93% identity with the human VEGFR-2 gene. Taqman quantitative RT-PCR demonstrated no statistically significant difference in levels of the gene expression in the jejunum comparing term animals with preterm animals (mean fold change, 1.3; 95% confidence intervals, 0.8 to 2.1; $n = 5$). The transcript was localized using *in situ* hybridization and was observed in the lamina propria of the villous core (Fig. 4). Localization of VEGFR-2 protein using radioligand binding with ^{125} I-VEGF confirmed binding of VEGF-A in the lamina propria of the villous core (Fig. 5). This could be displaced by an excess of unlabeled VEGF-A but not by PlGF, consistent with binding to VEGFR-2.

Immunohistochemistry using an antibody against the endothelial cell antigen, von Willebrand protein, demonstrated staining in the villous core (Fig. 6). The number of endothelial cells was compared between five preterm animals and four animals delivered at term using quantitative microscopy. The volume fractions of endothelial cells were 5.51% (SEM 0.87) and 5.97% (SEM 1.13), respectively ($p = 0.8$). The SD were 1.95 and 2.26. Taking a mean volume fraction of 5.51 and

assuming the same SD, we estimated that we would require 31 preterm and 31 term sheep to have 90% power to detect a 50% increase in the volume fraction of endothelial cells. Hence, the present study was underpowered to exclude a significant increase in the volume fraction of endothelial cells in this tissue with advancing gestational age.

DISCUSSION

This study demonstrates for the first time that VEGF-A and its receptor, VEGFR-2, are expressed in the fetal ovine small intestine during late gestation. Levels of VEGF-A increased between 130 and 145 d of gestation in parallel with the prepartum rise in fetal plasma cortisol concentration. When this increment in cortisol was abolished by fetal adrenalectomy, the prepartum increase in VEGF-A abundance in the fetal small intestine was abolished. Conversely, increasing fetal cortisol levels to prepartum values by exogenous infusion in the preterm fetus elevated VEGF-A abundance in the fetal intestine prematurely. Taken together, these data show that intestinal VEGF-A abundance is cortisol dependent during late gestation in the sheep fetus. This action of cortisol is in keeping with its other known maturational effects *in utero* (12) and suggests that VEGF-A is involved in the preparation of the fetal intestines for birth.

Figure 5. Receptor autoradiography using ^{125} I VEGF-A in fetal sheep ileum. (A) Dark field, low power. (B) Dark field of low power incubated with 25-fold excess of VEGF-A. (C) Dark field, medium power with 25-fold excess of PlGF. (D) High power of villous core of hematoxylin and eosin stained section. Bar = 100 μ m.

Figure 6. High power of immunohistochemistry using antibody against von Willebrand protein. Bar = 100 μ m.

The level of VEGF-A protein expression was statistically significantly greater among term animals compared with preterm animals, whereas the difference in gene expression was not statistically significant. Given the trend toward higher levels of gene expression in term animals, the absence of a statistically significant difference may simply reflect that the numbers were too small to achieve statistical significance given the variability among animals. Alternatively, it may reflect variation in the translation of mRNA for VEGF-A into protein comparing preterm and term animals. When we quantified the volume fraction of endothelial cells and compared preterm and term animals, there was no statistically significant difference. However, there was a great deal of variability in the measurement between animals. A power calculation using the observed mean volume fraction of endothelial cells demonstrated that we would have required a total of 31 preterm animals and 31 term animals to have 90% power to detect a 50% increase in the volume fraction of endothelial cells with advancing gestation. Further studies will be required to determine whether changes in VEGF-A are mirrored by significant changes in the extent of the capillary bed. This may be achieved by studying greater numbers of animals, or possibly by application of methods which allow improved assessment of the vascular tree, such as perfusion fixation (13).

Little is known about the role of VEGF-A in the intestines either before or immediately after birth. Inhibition of VEGF-A expression during the neonatal period in the mouse impairs growth and affects total body weight and organ growth and has effects on the liver, heart, and kidneys, with adverse consequences for neonatal viability (14). That study did not investigate the intestines. One previous study had investigated the expression of VEGF-A and its receptors in the intestine of the human neonate (15). It described VEGF-A in the endothelium of mesenteric vessels but not in the intestinal epithelium, whereas the VEGF-A receptor was detected in the glandular epithelial cells. However, only three infants were studied, all of whom were born preterm (25, 28, and 30 wk). Moreover, the antibodies used in this study to localize the receptors are known to lack specificity. To our knowledge, there are no other published data on the expression or regulation of

VEGF-A and its signaling pathways in the intestines during the perinatal period.

VEGF-A is a mitogen for endothelial cells from arteries, veins, and lymphatic vessels (3). It stimulates angiogenesis through a number of mechanisms but its main action is mediated by the VEGFR-2 receptor (16). This receptor binds VEGF-A with a K_d in the sub-nanomolar range and activates a cascade of mitogenic, anti-apoptotic, and chemotactic actions by phosphorylation of key regulatory proteins. In the present study, the VEGFR-2 receptor was localized to the lamina propria of the villous core of the fetal small intestine, a region containing an arteriole, a venule, a capillary network, and a lacteal. Large molecules absorbed across the intestinal mucosa, generally lipid, are transported *via* the lacteals, whereas smaller molecules cross into the capillary network and the venule. Increased intestinal VEGF-A protein expression in late gestation may, therefore, stimulate development of the villous microvascular and/or lymphatic systems in preparation for the transition from parenteral to enteral nutrition that occurs at birth. Therefore, preterm birth before the cortisol induced increase in VEGF-A expression may explain, in part, some of the intestinal complications associated with prematurity.

The most severe gastrointestinal complication of prematurity is NEC, which occurs with an incidence of 1–3 per 1000 live births and has a mortality rate of 25–40% (17). Its primary initiating factor in its pathophysiology is believed to be mucosal ischemia (1). The observation that the risk of NEC is reduced by half after antenatal glucocorticoid treatment (18) supports our suggestion that infants born preterm lack the cortisol-induced, VEGF-A-mediated stimulation of intestinal angiogenesis and, hence, suffer from mucosal ischemia and malabsorption. The current findings, therefore, raise the possibility of administering exogenous VEGF-A to the preterm human infant to reduce the incidence of intestinal dysfunction related to prematurity. This potential therapeutic treatment is consistent with the observation that VEGF-A levels are two orders of magnitude higher in human breast milk than serum (15).

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