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Tissue Factor Inflammatory Response Regulated by Promoter Genotype and p38 MAPK in Neonatal vs. Adult Microvascular Endothelial Cells

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Abstract

Objective and design—Variable tissue factor (TF) expression by human microvascular endothelial cells (HMVEC) may be regulated by two promoter haplotypes, distinguished by an 18 base pair deletion (*D*) or insertion (*I*) at -1208.

We sought to determine the relationship between these haplotypes and interleukin-1 (IL-1 α)-induced TF expression in neonatal versus adult HMVEC.

Results—IL-1-stimulated TF mRNA, protein, and activity were significantly higher in neonatal compared to adult *D/D* donors. IL-1-stimulated HMVEC from neonatal *D/D* donors expressed 3-fold higher levels of TF mRNA, 2-fold higher TF protein, and 4-fold increased TF activity compared to HMVEC from adult *D/D* donors. These results indicate that homozygosity for the *D* haplotype is characterized by increased response to IL-1 in neonates but not adults. IL-1 induced increased phosphorylation of p38 mitogen-activated protein kinase (MAPK), which was significantly greater in neonatal compared to adult HMVEC. Moreover, inhibition of the p38 MAPK pathway reduced IL-1-stimulated TF mRNA expression in *D/D* neonatal but not adult HMVEC.

Conclusions—Up-regulation of *D/D* neonatal HMVEC TF expression by IL-1 is mediated through the p38 MAPK pathway. This heightened response of *D/D* neonatal HMVEC to inflammatory stimuli may contribute to increased microvascular coagulopathies in susceptible newborn infants.

Keywords

Neonatal hemostasis; Microvascular coagulopathy; p38 MAPK; Tissue factor expression; Promoter haplotypes

Introduction

Neonatal hemostasis is developmentally immature compared to that of older children and adults. Although seemingly not a risk for most healthy infants, this maturational decrease in

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Conflict of Interest Disclosures

The authors declare no competing financial interests.

functional coagulation proteins, predisposes the sick newborn to either hemorrhagic [1–2] or thromboembolic [3] complications during stress, such as bacterial sepsis or traumatic injury.

Such conditions can trigger the release of inflammatory cytokines, which activate vascular endothelial cells (VEC), providing a pro-coagulant surface [4]. The pro-inflammatory cytokines, interleukin-1 (IL-1) and tumor necrosis factor- α (TNF- α), are produced by activated monocytes and EC. These cytokines induce Type II activation of the vascular EC [5], characterized by increased expression of tissue factor (TF) [6], the principal initiator of coagulation via the extrinsic pathway [7], other cytokines, such as IL-1, IL-6, IL-8, IL-11, SCF, and GM-CSF, [8–9] and adhesion molecules, such as CD40, E-selectin, and ICAM-1 [9–10]. At the same time, there is decreased expression of the coagulation inhibitors, thrombomodulin (TM) [11], tissue factor pathway inhibitor (TFPI) [12], and thrombospondin (TSP) [13].

A prior study has compared the expression of TF in HUVEC and adult saphenous vein EC (HSVEC) [14]. EC-bound TF activity was twice as high in IL-1-activated HUVEC compared to HSVEC, but no differences in TF mRNA expression were observed. Two major TF promoter haplotypes exist at essentially identical frequencies and are distinguished by three diallelic single nucleotide polymorphisms (SNPs), -1812C/T, -1322C/T and -603A/G, and an 18 bp insertion (*I*) or deletion (*D*) at -1208 [15–16], all of which are in complete linkage disequilibrium. Other investigators have clearly demonstrated association of homozygosity for the *D*-haplotype (-603A/-1208D/-1322C/-1812C) with increased TF production in IL-1-activated neonatal HUVEC [16]. Thus, altered expression of inflammatory response genes, such as TF, may contribute to the dysregulation of neonatal hemostasis as well as impaired host defense [11, 17].

EC from vessels of differing size or tissue origin are functionally heterogeneous [18], somewhat confounding interpretation of previous studies comparing EC from neonates and adults. The availability of primary dermal HMVEC from both neonates and adults provides a more appropriate model to compare the ability to support coagulation. Both EC types are derived from vessels of identical size and tissue origin, which are particularly susceptible to microvascular coagulopathic complications of sepsis [19–20]. In this study, we developed a primary HMVEC tissue culture model, consisting of both blood and lymphatic microvascular EC, to compare the IL-1-induced changes in expression of TF between neonatal and adult HMVEC.

Materials and Methods

Endothelial Cell (EC) Culture

Proliferating cultures of dermal HMVEC (third passage, 15–18 population doublings) were obtained from Lonza-Walkersville Inc. (Walkersville MD). The neonatal HMVEC were derived from foreskin tissue and the adult from non-pathological, elective surgical procedures. The average age of the adult donors utilized was 46 ± 4 years. The doubling times were not significantly different for the neonatal (24 ± 2 hrs) and adult (21 ± 2 hrs) HMVEC. Since the neonatal HMVEC are available from males only, we have obtained and analyzed adult HMVEC from both male and female donors, without observing any significant sex-based differences in their response to IL-1 (data not shown). HMVEC cultures were maintained in endothelial/microvascular growth medium-2 (EGM-2-MV, Lonza-Walkersville Inc., Walkersville MD). Experiments were performed during the fifth to eighth passage with neonatal and adult HMVEC pairs of comparable total population doublings after cultures reached approximately 80% confluence, as in previous studies [9]. All experiments compare primary neonatal vs. adult HMVEC from at least three different donor pairs.

These primary HMVEC have been reported to consist of 60–80% lymphatic endothelial cells (LEC) and the remainder blood endothelial cells (BEC) [21]. The relative proportion of each cell lineage in the HMVEC preparations was assessed by microarray analysis using vascular endothelial growth factor-C (VEGF-C) as a marker for BEC and podoplanin (PDPN) as a marker for LEC [22–23]. We found no significant differences in the PDPN/VEGF-C expression ratios ($p > 0.05$) between the neonatal and adult HMVEC preparations (Table 1). There was also no significant correlation between TF expression and the PDPN/VEGF-C expression ratios of the HMVEC TF genotypes ($p > 0.05$).

Cultures were exposed to optimized doses of 50 U/ml (300 pg/ml) IL-1 (R&D Systems Inc., Minneapolis MN) and inhibitors of the p38 MAPK (10 μ M SB-203580; Enzo Life Sciences Inc., Farmingdale, NY), NF- κ B (5 μ M BAY 11-7082; Enzo Life Sciences Inc., Farmingdale, NY), SAPK/JNK (2 μ M SP600125; Enzo Life Sciences Inc., Farmingdale, NY) or p44/42 MAPK (10 μ M U-0126; Cell Signaling Technology Inc., Danvers, MA) pathways.

mRNA expression analysis

Total cellular RNA was isolated using the PerfectPure RNA Cultured Cell Kit (5-Prime Inc., Gaithersburg, MD) from neonatal and adult HMVEC after preincubation for an optimized 1 hr. $\pm$ pathway inhibitor, and stimulation for an optimized 2 hrs. $\pm$ IL-1 α . Real-time RT-PCR was performed with the one-step, RNA Master SYBR Green I Kit, using 2.5mM Mn(OAc)₂, on a LightCycler 1.2 Real-Time PCR System (Roche Diagnostics Corp., Indianapolis IN). Previously described primers (0.6 μ M of each) specific for human TF [24] and β_2 -microglobulin (β_2 m), as a reference standard [25], generated 407 and 114 bp amplicons, respectively. HMVEC RNA (0.25 μ g per reaction) was reverse transcribed at 61°C for 20 min and then denatured at 95°C for 2 min, followed by 45 amplification cycles of 95°C denaturation for 5 sec, 60°C annealing for 5 sec, and 72°C extension for 15 sec, with a 65°C post-amplification melting curve target temperature. The cDNA clones, pHTF12 (courtesy of Dr. J.E. Sadler, Washington Univ.-St. Louis) [26] and pCMV-SPORT6:h β_2 m (Life Technologies Corp., Grand Island NY), were utilized as calibrators for LightCycler Relative Quantification (Roche Diagnostics Corp., Indianapolis IN) of TF and β_2 m amplification, respectively. Hybridization with human cDNA SH production microarrays by the Stanford Functional Genomics Facility (Palo Alto CA) essentially as described [27], was utilized to determine expression of vascular endothelial growth factor-C (VEGF-C) and podoplanin (PDPN) mRNA, as markers for BEC and LEC, respectively [22]. Normalized fluorescence was used to measure mRNA in triplicate cDNA hybridizations for each gene.

TF ELISA and activity assays

Confluent cultures of HMVEC were harvested at an optimized 24 hrs. after activation with IL-1 (R&D Systems Inc., Minneapolis MN) by three freeze–thaw cycles (–80°C room–temperature) in Tris-buffered saline supplemented with protease inhibitors (25mM Tris, pH=7.5, 137mM NaCl, 3mM KCl, 0.5mM EDTA, 0.1mM PMSF, 2 μ g/mL leupeptin, 5 μ g/mL pepstatin A, 10 μ g/mL aprotinin; Sigma-Aldrich Co., St. Louis MO). The IMUBIND-TF ELISA and Actichrome TF Assay (American Diagnostica Inc., Stamford CT) were utilized according to the recommendations of the manufacturer to determine induction of TF protein and activity, respectively.

Immunoblot analysis

Confluent cultures of HMVEC were harvested at an optimized 30 min after activation with IL-1 $\pm$ SB-203580 preincubation for an optimized 60 min, in RIPA buffer supplemented with both the protease inhibitors described above and phosphatase inhibitors (3mM sodium vanadate, 1 μ M okadaic acid, 10 mM sodium phosphate; Sigma-Aldrich Co., St. Louis MO),

as described [9]. The whole-cell extract proteins were electrophoresed on denaturing, SDS-polyacrylamide gels (12%, 1:37.5 bis:acrylamide; Bio-Rad Life Science Research, Hercules CA), along with BlueRanger Prestained Protein Molecular Weight Markers (Thermo-Fisher Scientific Inc., Rockford IL). Proteins were electroblotted onto nitrocellulose membranes using a MiniTrans-Blot Electrophoretic Transfer Cell (Bio-Rad Life Science Research, Hercules CA), according to the manufacturer's recommendations. Electroblot membranes were incubated with 5% nonfat milk in PBS for 60 min at room temperature, followed by 1:1000 anti-phospho-Thr180Tyr182-p38 MAPK, anti-p38 MAPK, anti-phospho-Thr334-MAPK-activated protein kinase-2, anti-phospho-Thr202/185Tyr204/187-ERK1/2, or anti-phospho-Ser63-c-Jun (Cell Signaling Technology Inc., Danvers MA) overnight at 4°C, and 1:1000 horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology Inc., Santa Cruz CA) for 2 h at room temperature. Membranes were washed with antibody binding buffer (2% nonfat milk in PBS) for 15 min at room temperature between incubations and with 0.1% Triton X-100 in PBS for 10 min × 3 before developing with ECL-Plus (GE Healthcare Bio-Sciences Corp., Piscataway NJ) chemiluminescent detection system and exposing to X-ray film. Following autoradiography, optical densities of the chemiluminescent signals were quantified by two-dimensional densitometry using the Doc-It Image Acquisition System (UVP Inc., Upland CA) and Scion Image (Scion Corp., Frederick MD).

Promoter genotyping

HMVEC genomic DNA was isolated using the Gentra PureGene Cell Kit (QIAGEN Inc., Valencia CA). A custom, complementary hybridization-based analysis for the -1322 C/T single nucleotide polymorphism (SNP) [15] was conducted using a Nanochip Molecular Biology Workstation (Epoch Biosciences Inc., Bothell WA). The following primers were chosen to amplify the -1423 to -1011 TF promoter region encompassing the SNP at -1322 [26]: 5'-GCATAAAAAGA-GGATTGCAT-3' and 5'-Bio-GCTGTAAACTTGCCAAGCAT-3'. HMVEC genomic DNA was amplified using one cycle at 94°C for 3 minutes, followed by 35 cycles of 94°C denaturation for 1 minute, 54°C annealing for 1 minute, and 72°C extension for 1 minute, with a 7 minute 72°C final extension. Amplicons were anchored to the Nanochip through biotin-streptavidin interactions for hybridization analysis. Detection utilized a combination of labeled *D*-allele (5'-Cy3-GCAACTTTCTGC-3') and *I*-allele (5'-Cy5-GGCAACTTTCTGT-3') hybridization reporters, as well as a stabilizer (5'-TTCGAGGAA-GGAAAGTCTACAAGGGCTAAACCTGG-3'), complementary to the sequence immediately 5' of the -1322 SNP, to neutralize any potential secondary structure. Synthetic oligonucleotides corresponding to both the *D*-allele (5'-Bio-CACATCCAGGTT-TAGCCCTTGTAAGTTTCCTTCCTCGAAGCAGAAAGTTGCCCTTGAT-3') and *I*-allele (5'-Bio-CACATCCAGGTTTAGCCCTTGTAAGTTTCCTTCCTCGAAGCAGAAAGTTGCCCTTGAT-3') forms of the -1322 SNP were also synthesized and biotinylated to serve as homozygous and heterozygous controls for assay validation. TF promoter amplicons were analyzed at an optimized discrimination temperature of 45°C as previously described [28]. The DNA haplotype indicated by -1322 C/T SNP analysis was confirmed using restriction enzyme testing of the -1423 to -1011 amplicon for an AluI site within the 18 bp promoter insertion/deletion region (data not shown).

Signal transduction pathway profiling

Total RNA isolated from IL-1 α -activated neonatal and adult HMVEC, as above, was reverse transcribed, labeled, and hybridized to Signal Transduction PathwayFinder Q-Series GEArrays as recommended by the manufacturer (QIAGEN Inc., Valencia CA). After exposure of washed macroarrays to X-ray film, signal optical densities were quantified by

two-dimensional densitometry using ScanAlyze and compared using GEMArray Analyzer Software (QIAGEN Inc., Valencia CA). The latest pathway linkages included in the GeneCards Human Gene Database V3 (www.genecards.org) were utilized to verify marker gene pathway associations[29].

Statistical analysis

Data are presented as the mean $\pm$ standard-error-of-the-mean (SEM) for three or more samples. Probability of significant differences ($p < 0.05$) between two data sets was calculated using Student's t-test, or among multiple data sets using one-way or Kruskal-Wallis analysis of variance, on InStat for Windows (GraphPad Software Inc., La Jolla CA). Probability of significant correlation ($p < 0.05$) between two data sets was determined using the Pearson test for linear correlation on InStat for Windows (GraphPad Software Inc., La Jolla CA).

Results

TF Expression in IL-1 α -Activated Neonatal vs. Adult HMVEC

The IL-1-induced expression of five EC-generated factors, TF, TSP, TFPI, TM and von Willebrand factor (VWF), was compared between neonatal and adult HMVEC. No significant differences were observed in the expression levels of TSP, TFPI TM or VWF (data not shown). The equivalent expression of these four genes following IL-1 induction indicated that both the neonatal and adult HMVEC were capable of mounting comparable inflammatory responses to IL-1 activation. However, TF mRNA abundance was increased in neonatal vs. adult HMVEC by 2- to 4-fold. After further analysis, variability of this increase was found to depend at least partially upon the TF promoter genotype (Figure 1). In response to IL-1 α , neonatal HMVEC homozygous for the *D* haplotype (*D/D*) expressed 3-fold higher levels of TF mRNA than adult *D/D* HMVEC ($p < 0.05$). Since basal expression of TF mRNA in unstimulated HMVEC was undetectable, TF mRNA induction by IL-1 was normalized to that of *I/D* adult HMVEC in each experiment for comparison (Figure 1). When the *D/D*, *I/D*, and *I/I* haplotypes of neonatal HMVEC were compared to *I/D* adult HMVEC, IL-1 α -induced TF mRNA expression was significantly increased ($p < 0.02$) only in the *D/D* neonatal haplotype (Figure 1). Furthermore, IL-1 α -induced TF mRNA expression was significantly less ($p < 0.05$) in *I/I* vs. *D/D* neonatal HMVEC (Figure 1). While IL-1 α -induced TF mRNA expression in *I/D* neonatal HMVEC appeared to be intermediate between that of the *I/I* and *D/D* haplotypes (Figure 1), it was not significantly different from either. These findings provide a possible explanation for the observed variability in IL-1-induced TF expression among neonatal HMVEC.

Despite persistent efforts to obtain *I/I* adult HMVEC, we were unfortunately unable to do so for this study. Nevertheless, although *I/I* adult HMVEC would have provided a full comparison, it did not seem likely that they would alter the findings centered on the *D/D* haplotype. Therefore, it was decided to proceed without them.

Since the difference in IL-1 α -induced TF mRNA expression between neonatal and adult HMVEC was significant for the *D/D* haplotype, further studies were conducted to determine if this differential expression was reflected in the expression and activity of the TF protein, as well. Comparable increases in TF protein (2-fold) and activity (4-fold) were observed in cytoplasmic extracts from IL-1 α -activated neonatal vs. adult *D/D* HMVEC ($p < 0.05$, in each case) (Figure 2). The apparent 2- to 3-fold higher levels of active (Figure 2B) compared to immunoreactive (Figure 2A) TF in both neonatal and adult HMVEC extracts may be due to its phospholipid milieu. Acidic phospholipids are required for TF activity[7],

and the native phospholipid content of the HMVEC extracts is likely to be more favorable for active TF than that of the relipidated activity assay standard.

Signal Transduction Pathway Profiling of IL-1 α -Activated Neonatal vs. Adult HMVEC

To investigate potential differences in the inherent response of neonatal HMVEC to inflammatory signaling that may contribute to the specific amplification of *D/D* TF expression, PathwayFinder macroarrays (QIAGEN Inc., Valencia CA) were used to compare the expression of 96 marker genes to assess the potential activation of 20 fundamental signal transduction pathways (Figure 3). Since expression of any individual gene may be induced through a variety of signal transduction pathways, activation of any single gene by a particular agonist cannot be unequivocally attributed to a specific pathway. However, the more genes known to be activated through a given pathway that are found to be induced by a particular agonist, the more likely that their activation is regulated through that given pathway. Therefore, up-regulation of these marker genes by IL-1 was compared in neonatal vs. adult HMVEC without regard for TF promoter genotype, to determine if there was an inherent difference in the neonatal HMVEC signal response to IL-1 that was contributing to the amplification of *D/D* TF expression,

Macroarray hybridization signals from 5 pairs of IL-1 α -activated neonatal and adult HMVEC, with various TF promoter genotypes, were compared using the GEArray Software provided by the manufacturer. Genes were considered to be differentially expressed based upon calculation of statistical significance ($p < 0.05$), as well as an experimentally imposed threshold of at least a 2-fold differential. Marker genes known to be activated by six of the 20 signal pathways represented were found to be significantly up-regulated by IL-1 α . The proportion of the up-regulated marker genes associated with each of these mitogen-activated protein kinase (MAPK), NF- κ B, Jak/STAT, PI3K/Akt/mTOR, PKA/cAMP, and Wnt pathways were then calculated and compared in the neonatal and adult HMVEC. Marker genes associated with MAPK, NF- κ B, or Jak/STAT pathway activation represented at least 20% of the total up-regulated in neonatal compared to adult HMVEC (Figure 3A). By contrast, only marker genes associated with NF- κ B pathway activation represented more than 20% of the total up-regulated in adult compared to neonatal HMVEC (Figure 3B). Consequently, the proportion of NF- κ B up-regulated genes was most similar in both neonatal and adult HMVEC. Although marker genes associated with Jak/STAT pathway activation represented somewhat less of the total up-regulated in the adult (15%, Figure 3B) vs. the neonatal (27%, Figure 3A) HMVEC, the most striking difference was the remarkable reduction in the proportion of MAPK-inducible marker genes from 20% in the neonatal (Figure 3A) to just 5% in the adult (Figure 3B) HMVEC.

These results indicated that IL-1 may trigger a more robust activation of a MAPK pathway in neonatal HMVEC. Although not definitive, these findings provided a basis for the following studies to further delineate the potential role of differential MAPK pathway activation in up-regulating TF expression specifically in neonatal HMVEC.

MAPK Pathway Contribution to Up-regulated *D/D* TF Expression in IL-1 α -Activated Neonatal HMVEC

The MAPK family plays a major role in endothelial cell stress and inflammatory signal response [30]. Both the MAPK and NF- κ B pathways contribute to regulation of TF by endothelial cells in response to inflammatory mediators[31]. We tested whether inhibiting any of the three key MAPK pathways, stress-activated protein kinase/Jun-N-terminal protein kinase (SAPK/JNK)[32], p44/42 (ERK1/2, MEK-ERK)[33], and p38[34], or the NF- κ B pathway[33] could reduce up-regulation of TF mRNA in IL-1 α -activated *D/D* neonatal HMVEC.

Pre-incubation of neonatal and adult HMVEC with inhibitors of the SAPK/JNK (SP600125) [33] or p44/42 (U-0126) [34] MAPK pathways prior to IL-1 stimulation had no significant effect on TF mRNA induction, whereas pre-incubation with an NF- κ B activation inhibitor (BAY 11-7082) [33] significantly abrogated TF mRNA induction ($p < 0.05$) equivalently in both neonatal and adult (49% and 54%, respectively) HMVEC (Figure 4). On the other hand, pre-incubation with an inhibitor of the p38 MAPK pathway (SB-203580) [34] had a significantly greater effect on IL-1 α -induced TF mRNA in *D/D* neonatal than adult HMVEC ($p < 0.01$), reducing its abundance by 58% vs. 20%, respectively (Figure 4). Inhibition of MAPK activity was verified by immunoblot analysis of the phosphorylated substrates, MAPK-activated protein kinase-2 (MAPKAPK-2), extracellular signal-regulated kinase 1/2 (ERK1/2), and c-Jun, for the p38, p44/42, and SAPK/JNK pathways, respectively (Table 2). NF- κ B inhibition was not subjected to this additional analysis, since TF mRNA expression was significantly inhibited by BAY 11-7082 equivalently in both neonatal and adult HMVEC (Figure 4). The phospho-MAPKAPK-2, -ERK1/2, and -c-Jun MAPK pathway substrates were all significantly reduced equivalently in both neonatal and adult HMVEC following incubation with their respective inhibitors (Table 2). The significant difference ($p < 0.01$) in the inhibition of TF mRNA expression between *D/D* neonatal and adult HMVEC by SB-203580 (Figure 4) suggested that a more robust response of the p38 MAPK pathway to IL-1 in neonatal HMVEC may activate a *D/D* promoter-specific mechanism to amplify TF mRNA expression.

Finally, phospho-p38 immunoblotting was also employed to assess whether p38 MAPK was differentially activated by phosphorylation in response to IL-1. IL-1 was found to induce a 5.8-fold increase in p38 MAPK phosphorylation in neonatal compared to 1.9-fold in adult ($p < 0.04$) HMVEC (Figure 5).

Taken together, these results suggest that the p38 MAPK pathway is hyperactivated by IL-1 in neonatal HMVEC. Hyperactivation of the neonatal HMVEC p38 MAPK pathway may up-regulate a *D/D* promoter-specific mechanism, which amplifies TF mRNA expression in neonatal, but not adult, HMVEC.

Discussion

Reduced levels of anti- and pro-thrombotic proteins are characteristic of neonatal hemostasis [1–2]. Consequently, sick newborns are particularly susceptible to microvascular coagulopathies such as disseminated intravascular coagulation (DIC), with over half of all pediatric cases occurring during the neonatal stage [35]. Decreased production of the inhibitors such as antithrombin and protein C by the immature liver is believed to contribute to a 10-fold greater thrombotic risk in newborns compared to older children. Premature infants have even lower levels of these coagulation inhibitors and have an even higher risk of DIC with infection or hypoxia. There is also evidence that TF may contribute to the initiation of DIC [7]. However, the mechanism behind the 10-fold increased risk for thrombosis in neonates has yet to be definitively established [36]. The increasingly clear connections of TF expression [7], with inflammation [5], microvascular coagulopathies [35], and promoter haplotype in EC [16] led us to investigate the response of TF to IL-1 in neonatal HMVEC.

The variability of TF expression among neonatal HMVEC led us to investigate the possible contribution of TF promoter genotypes previously shown to play a role in TF regulation by Terry and coworkers [16]. Their study found that neonatal HMVEC homozygous for an 18 bp deletion at -1208 (*D/D*) expressed much higher levels of TF in response to IL-1 than those homozygous (*I/I*) or heterozygous (*I/D*) for the 18 bp insertion, as previously described in HUVEC [16]. Several studies have found modest but significant elevations in

circulating TF [15] and monocyte TF mRNA [37] by *I/I* vs. *D/D* human subjects. However, this differential expression was not always significant following endotoxin activation [37]. Furthermore, none of these clinical studies could address EC expression nor compare TF expression in response to IL-1. The results reported herein showed that neonatal *D/D* HMVEC expressed significantly higher levels of TF mRNA than the *I/D* or *I/I* genotypes in response to IL-1 (Figure 1), corroborating the previously reported findings for *D/D* HUVEC by Terry et al.[16]. Remarkably, the present study found that IL-1-stimulated *D/D* neonatal HMVEC also expressed significantly more TF mRNA than *D/D* adult HMVEC (Figure 1), which was not previously compared[16], as well as TF protein (Figure 2A) and activity (Figure 2B).

These findings suggested that there may be a different response to IL-1 in the neonatal HMVEC unique to the *D* haplotype. For example, an alternative pathway may be up-regulated by IL-1 only in neonatal HMVEC that then activates a transcription factor specific for the *D* haplotype. To test this hypothesis, PathwayFinder macroarrays were used to compare IL-1 α -induction of 96 marker genes representing 20 signal transduction pathways in neonatal vs. adult HMVEC[29]. This analysis showed that while the MAPK, NF- κ B, Jak/STAT, PI3K/Akt/mTOR, PKA/cAMP, and Wnt pathways appeared to be activated by IL-1 in both neonatal and adult HMVEC, the proportion of MAPK up-regulated marker genes was considerably greater in neonatal HMVEC (Figure 3).

The MAPK[30] and NF- κ B[38] pathways are known inflammatory regulators of TF expression. While there is little existing evidence of a role for Jak/STAT activation regulating TF in response to inflammatory agonists, a Jak/STAT-mediated response to IL-1 following initial MAPK activation has been reported[39]. Since MAPK activation was the most evident difference between the response of neonatal and adult HMVEC to IL-1 (Figure 3) and is central to the inflammatory response of EC[30–31, 39], the potential contributions of the three key MAPK signal pathways to *D/D* TF up-regulation in neonatal HMVEC were evaluated through the use of specific inhibitors. NF- κ B inhibition was also utilized for comparison[38]. Inhibition of the SAPK/JNK or p44/42 MAPK pathways prior to IL-1 stimulation had no significant effect on induction of TF mRNA, whereas inhibition of NF- κ B significantly abrogated the expression of TF mRNA equivalently in both neonatal and adult HMVEC (Figure 4). However, inhibition of the p38 MAPK pathway had a significantly greater effect on IL-1 α -induced TF mRNA expression in *D/D* neonatal compared to adult HMVEC (Figure 4). Phospho-Thr180/Tyr182-specific immunoblotting also found a significantly greater increase in p38 MAPK phosphorylation in neonatal compared to adult HMVEC (Figure 5). Taken together, these results suggest that the IL-1 induced p38 MAPK pathway is hyperactive in neonatal HMVEC. This may represent an alternative, IL-1-activated signal transduction mechanism, by which transcription of the *D* haplotype promoter is uniquely enhanced, leading to amplified TF mRNA expression.

A schematic model (Figure 6) summarizes the hypothetical response of the neonatal HMVEC TF gene to IL-1. The model depicts key components of the p38 MAPK and NF- κ B pathways. It should be noted that the specific steps of the p38 MAPK pathway responsible for its hyperactivation by IL-1 in neonatal HMVEC have not yet been determined. The model illustrates the potential up-regulation of one or more transcription factors specific for the *D* haplotype (hatched arrows). The earlier study by Dr. Terry and coworkers [16] also compared nuclear proteins in HUVEC that bind to either the *D* or *I* haplotype promoter. They found distinctly different protein complexes interacting with the -1208 promoter region in the presence or absence of the 18 bp insertion, but the proteins involved have yet to be identified. If this region serves as a distal enhancer element, the authors proposed that the 18 bp insertion at this position may disrupt transcription activator binding sites, leading to the decrease in mRNA expression observed in the *I* haplotype [16]. On the other hand, the

18 bp insertion was also found to introduce C/EBP and GATA transcription factor-binding motifs [16], which could then act as repressors to down-regulate transcription in the *I* haplotype, as observed for other genes [40–41]. The results of the present study indicate that IL-1 induces an amplified p38 MAPK signal in neonatal HMVEC (Figures 3, 4, & 5) supporting a *D* haplotype-specific transcription enhancer/activator model (Figure 6) for TF up-regulation (Figures 1&2), as proposed by Dr. Terry et al.[16]. Finally, even though the 18 bp insertion/deletion is the most conspicuous feature distinguishing these promoter haplotypes, clearly potential *cis*-acting elements containing any of the other three haplotype-specific polymorphisms [15] and associated *trans*-acting factors may indeed be responsible for differential TF regulation, as well.

Although there is evidence implicating endothelial TF as a primary initiator of vascular thrombosis[42], its association with DIC secondary to sepsis is less clear[43]. While other studies have contributed to the hypothesis that TF production by monocytes triggers DIC secondary to sepsis[44], there is also evidence that monocytic TF is not necessarily associated with DIC[45]. Since it appears likely that both of these cellular sources of TF may play a role, it will be interesting to investigate whether or not neonatal monocytes show a similar promoter haplotype dependency for TF expression. Our comparison of blood and lymphatic microvascular EC marker gene expression found that the proportion of these HMVEC lineages did not vary significantly among the neonatal and adult isolates used in this study (Table 1). Thus, while comparative regulation of TF in BEC vs. LEC was not the focus of this study, these marker gene findings indicated that comparison of TF regulation in our neonatal vs. adult primary HMVEC isolates remains statistically sound. Nevertheless, it will also be interesting to determine if there are any differences in TF expression by isolated BEC compared to LEC[23], especially since expression of TF by isolated LEC has not been well-characterized. Finally, this study sets the stage to expand the search[16] for key promoter elements and transcription factors regulating promoter haplotype-dependent TF expression in EC. Comparing regulation in *D/D* neonatal vs. adult HMVEC, as well as in response to p38 MAPK inhibition, will provide new tools to aid in elucidating the control mechanism.

In summary, the unique response of neonatal HMVEC that we describe here reflects a developmental transition from fetal to adult gene regulation, which significantly affects the neonate's response to inflammation and injury. From a developmental standpoint, TF likely plays a central role in the coordination of inflammatory and coagulation responses at the EC surface. The differences in IL-1 activation of neonatal vs. adult HMVEC provide a useful model to investigate and compare coordinated gene regulation. The results obtained in this model will provide new insights into developmental differences in signal transduction pathways that place the newborn at higher risk for microvascular coagulopathy during sepsis. For example, the TF promoter haplotype may eventually qualify as a genetic biomarker for thrombotic risk in susceptible *D/D* newborn infants.

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Figure 1. Neonatal HMVEC TF genotype-dependent IL-1 α -induced mRNA expression compared to I/D adult HMVEC

Total RNA isolated from primary HMVEC was analyzed by real time RT-PCR after stimulation for 2 hrs with 50 U/ml IL-1 α . TF mRNA expression by individual neonatal HMVEC genotypes was compared to that of I/D adult HMVEC, of equivalent cumulative population doublings, within each experiment. Basal expression of TF mRNA in unstimulated HMVEC was undetectable. The results are shown as mean $\pm$ SEM % of abundance in I/D adult HMVEC, and represent a compilation of eleven different experiments with three different sets of the individual genotypes. $^{\dagger} p < 0.05$ vs. D/D Adult HMVEC, $^{\ddagger} p < 0.02$ vs. I/D Adult HMVEC, $^* p < 0.05$ vs. I/I Neonatal HMVEC.

Figure 2. Neonatal vs. adult *D/D* HMVEC IL-1 α -induced TF protein abundance and activity
 Whole-cell, freeze-thaw, protein extracts were prepared from primary HMVEC after stimulation for 24 hrs with 50 U/ml IL-1 and assayed for (A) TF protein abundance ([†] $p < 0.03$) using the IMUBIND-TF ELISA Kit and (B) TF activity (* $p < 0.02$) using the Actichrome TF Kit, normalized to total protein content (20 μ g per sample), as determined by BCA Protein Assay (Thermo-Fisher Scientific Inc., Rockford IL). Basal TF protein expression and activity in unstimulated HMVEC was undetectable. The results are shown as mean $\pm$ SEM, and represent a compilation of three different experiments with three different sets of *D/D* HMVEC.

Figure 3. Macroarray analysis of IL-1 α -induced signal transduction pathway activation in neonatal compared to adult HMVEC

Total RNA isolated from primary HMVEC was used to reverse-transcribe, amplify, and ^{32}P -label cDNA with the optimized AmpoLabeling-Linear Polymerase Amplification Kit for hybridization after stimulation for 2 hr with 50 U/ml IL-1 α . PathwayFinder macroarray hybridization signals from 5 neonatal and adult HMVEC pairs of various TF promoter genotypes were compared using the GEMMA Software provided by the manufacturer. Marker genes were considered to be differentially expressed based upon calculation of statistical significance ($p < 0.05$), as well as an experimentally imposed threshold of at least a 2-fold differential. Pathway activation was calculated by comparing the fraction of total pathway marker genes up-regulated by IL-1, in neonatal vs. adult HMVEC (**A**), and adult vs. neonatal HMVEC (**B**), for marker genes known to be induced by each of the MAPK, NF- κ B, Jak/STAT, PI3K/Akt/mTOR, PKA/cAMP, and Wnt, pathways[29].

Figure 4. Neonatal vs. adult *D/D* HMVEC IL-1 α -induced TF mRNA abundance after signal pathway inhibition

Total RNA isolated from primary *D/D* HMVEC was analyzed by real time RT-PCR, after pre-incubation $\pm$ inhibitors of the NF- κ B, p38, p44/42, or SAPK/JNK MAPK signal pathways for 1 hr before stimulation for 2 hr with 50 U/ml IL-1 α . The results are shown as mean $\pm$ SEM % of uninhibited mRNA abundance, and represent a compilation of four different experiments with three different sets of *D/D* HMVEC. * $p < 0.01$ vs. uninhibited (100%), $\dagger$ $p < 0.05$ vs. *D/D* Adult HMVEC.

Figure 5. Immunoblot analysis of IL-1 α -induced p38 MAPK phosphorylation in neonatal compared to adult HMVEC

Cytoplasmic protein extracts were prepared from primary HMVEC after stimulation for 30 min $\pm$ 50 U/ml IL-1 α . Phosphorylated and total p38 MAPK were detected with anti-phospho-p38 MAPK and anti-p38 MAPK, respectively. The results are shown as mean $\pm$ SEM % of unstimulated phospho-p38 MAPK, after normalization to total p38 MAPK, and represent a compilation of three different experiments with three different pairs of neonatal and adult HMVEC of various TF promoter genotypes. * $p < 0.04$.

Figure 6. Model pathway for genotype-dependent TF induction in neonatal HMVEC

The major components of the NF-κB and p38 MAPK signaling pathways in response to IL-1 are diagrammed. The thicker arrows of the neonatal p38 MAPK pathway indicate its apparent increased activity in IL-1-activated neonatal HMVEC (Fig. 4). The hatched arrows represent potentially up-regulated *D/D* genotype-specific transcription factors that may preferentially induce the *D/D* TF promoter in response to p38 MAPK activation by IL-1 in neonatal HMVEC (Fig. 5). “X” indicates sites of inhibitor action. Pathway illustration template courtesy of QIAGEN Inc., (Valencia CA).

Proportion of Marker Genes Characteristic of Lymphatic (PDPN) vs. Blood (VEGF-C) EC Expressed by Neonatal (Nb) and Adult (Ad) HMVEC of Each TF Genotype Studied.

Table 1

PDPN / VEGF-C Expression Ratio	Nb-D/D	Nb-I/D	Nb-I/I	Ad-D/D	Ad-I/D
Mean±SEM	1.62±0.10	1.73±0.58	4.24±1.34	1.84±0.16	2.06±0.11
Range	1.46–1.81	0.63–2.57	1.57–5.80	1.64–2.15	1.88–2.25
Significance*	N.S.	N.S.	N.S.	N.S.	N.S.

* N.S. = Not Significant ($p > 0.05$), calculated using one-way analysis of variance (ANOVA), with Tukey-Kramer Multiple Comparison Post-Test.

Table 2

Substrate Phosphorylation Following Pathway-Specific Inhibition by Immunoblot Analysis. Neonatal and adult HMVEC of various TF promoter genotypes were IL-1 α -activated with and without pre-exposure to inhibitors.

Pathway/ Substrate	<u>Substrate Phosphorylation after Inhibition</u>	
	Neonatal	Adult
p38 MAPKAPK-2	9.87 $\pm$ 0.73% [‡]	7.04 $\pm$ 0.61% [‡]
p44/42 ERK1/2	13.13 $\pm$ 1.45% [†]	17.89 $\pm$ 2.34% [†]
SAPK/JNK c-Jun	31.24 $\pm$ 2.89% [*]	30.32 $\pm$ 6.76% [*]

Results represent mean $\pm$ SEM % compared to uninhibited substrate phosphorylation in response to IL-1 α .

* $p<0.05$; [†] $p<0.02$; [‡] $p<0.01$.