2-11-2008

Inflammatory and Thrombotic Responses to Microbial Products in Fetal Vessels Are Mediated through Divergent Toll-Like Receptor Signaling Pathways: Implications in Fetal Inflammatory Response Syndrome

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INFLAMMATORY AND THROMBOTIC RESPONSES TO MICROBIAL PRODUCTS IN FETAL VESSELS ARE MEDIATED THROUGH DIVERGENT TOLL-LIKE RECEPTOR SIGNALING PATHWAYS: IMPLICATIONS IN FETAL INFLAMMATORY RESPONSE SYNDROME.

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

By Shekar Ligia Davarya

2007
Placental vessels and the umbilical circulatory network function to carry oxygen and nutrients to the fetus. It is at this level that placental lesions such as villitis, obliterative vasculopathy, and thrombotic vasculopathy have been observed in association with fetal inflammatory response syndrome (FIRS) and cerebral palsy. We used human umbilical vein endothelial cells (HUVECs) as a model to study the regulation of inflammation and thrombosis in fetal vessels by microbial products. In this thesis we measured interleukin-8 (IL-8) and tissue factor (TF) expression by HUVECs treated with lipopolysaccharide (LPS), poly (I:C) (PIC), and peptidoglycan (PG). Our results show a profound induction of IL-8 by PIC, a TLR-3 ligand. We also show a moderate induction of tissue factor expression in PIC-treated HUVECs. These results show that HUVECs are exquisitely sensitive to PIC and suggests an important role for viral infection in umbilical vessel inflammation. We additionally treated HUVECs with dexamethasone (DEX), an anti-inflammatory steroid, and melatonin (MT), a pineal gland product with immunomodulatory and anti-oxidant properties. DEX reduced the level of both IL-8 and TF expression in PIC-treated cells. MT, however, further enhanced IL-8 expression in PIC-treated cells. Our results indicate a potential role for glucocorticoid therapy in reducing placental vessel inflammation and thrombosis. Thus, intervention with GC in pregnancies with FIRS may reduce the severity of placental lesions associated with cerebral palsy.
ACKNOWLEDGEMENTS

I would like to thank Dr. Seth Guller whose mentorship was essential to the development and completion of this work. I would also like to thank my husband for his unending support.
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INTRODUCTION

Cerebral Palsy

Cerebral palsy (CP) is a heterogeneous group of non-progressive sensorineural and motor deficit disorders with an incidence of 2-3 per 1000 live births (1). CP was thought to arise as a consequence of intrapartum asphyxia but current theories are implicating antenatal insults in the majority of CP cases (2). Additionally, some fetuses may be genetically more susceptible to sublethal antepartum insults and manifest with neurological deficits. Although the etiology and timing of CP-related events remains controversial, associated thromboinflammatory placental lesions have been identified. The placenta is the main barrier separating the fetus from exposure to microorganisms, toxins, and harmful immunologic reactions. Lesions occurring in the fetoplacental vasculature and associated inflammatory processes can contribute to reduced fetal perfusion and/or microbial access to fetal tissues.

Fetoplacental thromboinflammatory lesions are the result of subacute or chronic processes beginning days to weeks before delivery. A study by Redline in 2005 found that over 50% of placentas from CP and neurologically affected infants showed one or more thromboinflammatory lesions. This association is far greater than the presence of similar lesions in only 10% of control placentas examined (3). Examples of these thromboinflammatory lesions include fetal thrombotic vasculopathy, chronic villitis with obliterative fetal vasculopathy, and chorioamnionitis with fetal vasculitis (3, 4). Thrombotic vasculopathy describes the degeneration of fetal vascular beds of terminal villi resulting from prolonged occlusion of the supplying fetal placental vessels (5). This diagnosis can be made upon histological inspection showing 15 or more affected villi per
section (3). Chronic villitis with obliterate vasculopathy results from the destructive effects of maternal leukocytes which infiltrate fetal vascular tissue. The resulting villitis is often of unknown etiology but may accompany microbial infection. In the third lesion, chorioamnionitis is accompanied by a severe fetal inflammatory response and destructive vasculitis. Although relatively common, chorioamnionitis may be accompanied by an exaggerated inflammatory reaction characterized by a confluent neutrophilic infiltrate of large chorionic and umbilical vessels, also described as funisitis (6, 7). The resulting vasculitis can lead to impaired vascular integrity and formation of mural thrombi, reducing the vascular capacity of the placenta.

The precise mechanisms by which thromboinflammatory placental lesions may lead to cerebral palsy are not known. The placental vascular beds are in direct continuity with fetal circulation via the umbilical vessels. Inflammatory cytokines, vasoactive mediators, and activated immune cells involved in thromboinflammatory placental lesions can spread to downstream fetal tissues and contribute to a fetal inflammatory response syndrome (FIRS) (8). FIRS is described as diffuse endothelial damage and coagulopathy in portions of the fetal circulation, including the CNS, leading to tissue ischemia (8). In a study by Gomez et al., FIRS, defined as elevated fetal plasma interleukin-6, was identified as an independent risk factor for severe neonatal morbidity (8).

Toll-like Receptors

Microbial pathogens elicit innate inflammatory reactions in the placenta via specific Toll-like receptors (TLRs). The TLRs are membrane-associated protein receptors belonging to the superfamily of interleukin receptors. To date, thirteen
members of the TLR family have been identified, TLR1-13, where each recognize distinct pathogen-associated molecular patterns (PAMPs) via the extracellular domains (9). Of particular interest are TLR-2, -3, and -4 which specifically interact with bacterial and viral-derived PAMPs. Bacterial and viral infection make up the majority organisms responsible for intrauterine infections. TLR-2 recognizes bacterial peptidoglycan (PG), a component of Gram-positive cell wall. TLR-2 is critical in mounting innate immune responses to organisms such as *Staphylococcus aureus* and *Listeria monocytogenes*, a finding demonstrated by susceptibility of TLR-2 -/- mice to fulminant infections by these organisms (10). TLR-3 recognizes ds-RNA produced during viral infections (11). TLR-3 has additionally been shown to bind polyinosinic-polycytidylic acid (poly-IC), a synthetic viral ds-RNA analog (11). TLR-4 recognizes Gram-negative bacterial PAMPs such as lipopolysaccharide, a Gram-negative cell wall component. TLR-4 -/- infected with *Neisseria meningitidis* and *Escheria coli* had impaired immune responses specifically with neutrophil recruitment to the site of infection (12).

TLR-2 and -4 both transduce intracellular signals by recruiting and binding the adaptor protein MyD88 (9). MyD88 then associates with interleukin-1 receptor-associated kinase (IRAK) and promotes IRAK auto-phosphorylation. IRAK then dissociates from MyD88 and subsequently interacts with tumor necrosis factor receptor-associated factor-6 (TRAF-6). TRAF-6 then activates transforming growth factor-beta-associated kinase-1 (TAK-1) which, when complexed to TAK-1 binding protein (TAB-1), phosphorylates inhibitor-κB kinase (IKK). IKK then phosphorylates IκB which is subsequently degraded. Without a bound IκB molecule, cytoplasmic NF-κB dimers may freely translocate to the nucleus. Unlike TLR-2 and -4, TLR-3 mediates intracellular
signaling independent of MyD88 (13). Via a Toll/interleukin-1 receptor (TIR) domain, TLR-3 recruits and binds TIR domain-containing adaptor inducing interferon-β (TRIF). TRIF directly binds TRAF-6 to activate the TRAF-6 mediated signaling cascade described above. TLR-2, -3, and -4 mediated signaling all converge on the activation of the transcription factor NF-κB, a prominent mediator of inflammatory cytokines, chemokines, adhesion molecules, and acute phase reactants (14). NF-κB induces expression of molecules such as interleukin-1, -2, -6, -8, -12, and tumor necrosis factor-α (TNF-α) (5).

The current study will specifically explore roles of TLR-2, -3, and -4 in expression of inflammatory and thrombotic mediators in HUVECs. Although TLRs are critical in host innate immunity, an excessive inflammatory reaction mediated by these same pathways may be detrimental to the local placental vasculature and thus, the fetus. We will specifically measure the induction of IL-8 as a marker for inflammation. IL-8 is a potent neutrophil chemoattractant and may be a major contributor in fulminant neutrophilic infiltrates noted in chronic villitis and funisitis (15). IL-8 is additionally capable of mediating local pro-thrombotic events, described below, which may contribute to obliterative vasculopathy. Prior studies have demonstrated TLR mediated induction of IL-8 and other inflammatory cytokines in placental tissue (16, 17). Induction of IL-8 expression by a TLR-4 specific pathway has also been shown in human umbilical vein endothelial cells (HUVECs) (17). There are additional neutrophil chemoattractants, such as TNF-α, whose TLR-mediated expression may contribute to a neutrophil-rich inflammatory response. There is less evidence that TNF-α, versus IL-8, may be involved in pro-thrombotic signaling cascades in endothelial cells.
Tissue Factor

Tissue factor (TF) is an integral component of vascular homeostasis. The TF glycoprotein is expressed on the surface of endothelial cells in response to endothelial cell damage (18). Its presence initiates the extrinsic arm of the coagulation cascade and promotes downstream enzymatic activation of thrombin and fibrin, completing the formation of an insoluble clot (18). Increased TF expression has been noted in the setting of inflammation, a pro-thrombotic environment (18). The presence of LPS, viral infection, and inflammatory cytokines such as IL-8 can increase expression of inducible TF in endothelial cells (19, 20, 21). The connection between inflammation and thrombosis is not completely understood but some molecular mechanisms have been proposed. A prior study showed LPS-mediated TF induction was reduced in the presence of protease inhibitors which prevented IκB degradation and subsequent release of NF-κB. This suggests a role for NF-κB in TF expression, a mechanism which can involve TLR-mediated NF-κB activation (22). A binding site for NF-kB has also been identified in the human TF promoter region, further supporting the connection between NF-κB and TF expression (23). The association between thrombotic and inflammatory pathways may offer an explanation for the observation that chronic villitis often coexists with obliterative vasculopathy in the placenta.

Glucocorticoids

Glucocorticoids (GC) are endogenous adrenal hormones with activity in many cell types. GC is a lipophilic molecule which freely diffuses across the cell membrane to interact with its cytosolic GC receptor (GR). GC has been shown to modulate expression of several inflammatory genes including cytokines (IL-1, IL-6, TNF-α), chemokines (IL-
8, RANTES, MCP-1, -2, -3) and adhesion molecules (ICAM-1, VCAM-1, E-selectins) (24). Cytoplasmic GR has been localized to placental tissue including endothelial cells (25). Although the mechanism of GC action is likely multifold, there are two activities which decrease the production of inflammatory cytokines such as IL-8 (26). First, GC is thought to indirectly reduce pro-inflammatory gene expression by inducing expression of anti-inflammatory molecules. Once GC is bound to GR, the complex translocates to the nucleus where GR can interact with coactivator molecules: cAMP-response-element-binding protein (CBP) and p300/CREB-associated factor pCAF (24). GC complexes with CBP and pCAF on glucocorticoid response elements (GREs) and promotes acetylation of leucine residues on histones. Histone acetyltransferase (HAT) is an inherent activity of the CBP molecule. Via acetylation of histones and subsequent changes in chromatin wrapping, the DNA containing anti-inflammatory gene sequences becomes transcriptionally active. IκB is included in the complement of GC-induced proteins and its presence blocks NF-κB translocation to the nucleus and thus prevents NF-κB induced pro-inflammatory gene expression (27). The second activity of GC involves direct suppression of pro-inflammatory genes at the transcriptional level. GC-bound GR again translocates to the nucleus and selectively binds to CBP and pCAF in an NF-κB transcriptionally active region (24). GR then recruiites histone deacetylase-2 (HDAC-2) and reverses acetylation of histones, preventing chromatin rearrangement and subsequent transcription of NF-κB induced genes. These modulatory effects suggest a role for GC as an anti-inflammatory agent in umbilical vessels with potential therapeutic value in the setting of suspected funisitis. This study will specifically test the effects of GC on IL-8 expression in LPS/PIC/PG activated HUVECs as a model for anti-
inflammatory actions during pregnancy. We will also examine the effects on GC on HUVEC TF expression in conjunction with its potential thrombo-inflammatory action.

Melatonin

Melatonin (MT) is a pineal gland product with various immunomodulatory effects including anti-oxidant properties (28). Melatonin is expressed endogenously in mammals in diurnal peak-trough concentrations (29). The MT molecule freely diffuses across biological membranes including the blood-brain barrier (29). Of particular interest is the ability of MT to scavenge oxygen and nitrogen free-radicals independent of MT receptor activity (29). MT also possesses immunomodulatory signaling activity. MT has been shown in murine macrophage cells to inhibit activity of NF-κB (30). This MT activity has not yet been explored in umbilical endothelium and can be a second potential therapeutic agent for immune modulation. The actions of GC and MT may act in concert to deliver an optimal protective anti-inflammatory effect on the fetus and placenta.

HUVECs

HUVECs are endothelial cells derived from the umbilical vein which delivers oxygenated and nutrient-rich blood from the maternal to fetal circulatory system. The umbilical vein is the conduit through which ascending bacterial and viral infections can hematogenously spread to the fetus. The umbilical vein is also the site of funisitis, a fulminant vessel inflammation associated with poor neonatal outcome (3, 4). We have chosen HUVECs as an in-vitro model to approximate endothelial-cell mediated inflammatory responses in the umbilical vein in the presence of microbial products. Fetal endothelial cells are key sites of microbial-driven inflammation in FIRS (6-8). HUVECs are also a widely used in vitro model, and can be easily maintained and manipulated. The limitations of using this model include the inability to observe the multicellular vascular
environment in which pathologic vascular lesions have been described. Since HUVECs do not organize into discrete vessels under conditions chosen for investigation, we will not be able to correlate our observations with those noted by histologic inspection of placentas delivered in pregnancies with children that develop CP. Fetal vascular smooth muscle cells may also be a valuable model system in which to study the effects of infection on inflammation as they are known to be damaged in pregnancies with neonatal encephalopathy and CP (6-8). Although not currently available, fetal neural endothelial cells would also be valuable for direct analysis of the effects of microbial products on inflammatory cytokine expression in the CNS.
HYPOTHESIS

The working hypothesis is that microbial products differentially regulate inflammatory cytokine production in fetal vessels through TLR-specific pathways. Using human umbilical vein endothelial cells as a model, we will test the effects of bacterial and viral products on the expression of mediators of inflammation (i.e. cytokines) and thrombosis (i.e. TF). We also propose that GC and MT treatment promote anti-inflammatory and anti-thrombotic effects in HUVECs. The reduction of local inflammation and thrombosis would be expected to suppress fetal-placental inflammation associated with FIRS, and potentially the incidence of CP.

SPECIFIC AIMS

1. To test the hypothesis that TLRs differentially regulate cytokine expression in fetal vessels.
   
   a) HUVECs will be stimulated with LPS, PIC, or PG. IL-8 mRNA will be measured by quantitative RT-PCR. IL-8 protein production will be measured by ELISA.
   
   b) TF production will be measured using a TF specific ELISA

2. To test the hypothesis that GC and MT treatment decrease the production of pro-inflammatory and pro-thrombotic factors by HUVECs.
   
   a) HUVECs stimulated with LPS, PIC, or PG will be treated with GC or MT. Cytokine production will be measured using an IL-8 specific ELISA.
   
   b) TF production will be measured using a TF specific ELISA.
METHODS

Cell Culture

HUVECs were obtained from the American Type Culture Collection. Cells were grown in a humidified environment at 37ºC at 5% CO2/ 95% room air. HUVECs were subcultured into 24-well plates (Beckton Dickinson, Grand Island, NY) for IL-8 studies and T25 flasks (Beckton Dickinson) for TF and mRNA studies. HUVECs were maintained in medium containing 10% stripped FBS without hydrocortisone at least 48 hr prior to treatment. Cells at 80% confluence were stimulated with or without 0.1 ug/ml of LPS, PIC, or PG. Cells were simultaneously treated with or without 100 nM Dexamethasone (DEX) or 10 µM MT. Concentrations and incubation times for LPS/PIC/PG and DEX treatments were based on methods in previous studies demonstrating anti-inflammatory activity in HUVECs and other placental cells (16, 17). MT dosage and incubation time was adapted from studies in murine macrophages which showed anti-inflammatory activity (30). For conditions with dual DEX and MT treatment, 1nM DEX and 0.5 uM MT were used. Treatments were performed in either serum-free medium or medium containing 10% stripped FBS for 24 or 48h. Wells were washed twice with Dulbecco’s phosphate buffered saline (PBS) (Gibco, Grand Island, NY) prior to treatment in serum free conditions.

IL-8 Cytokine Assay

Levels of IL-8 in conditioned media were measured with an IL-8 specific ELISA (R&D Systems, Minneapolis, MN). Conditioned media from 24-well dishes were harvested at 24 and 48hr and added to 96-well dishes pre-coated with mouse anti-human IL-8 antibody. After 2h, wells were washed three times with wash buffer (0.05% Tween 20 in
PBS) and incubated with biotinylated goat anti-human IL-8 antibody for 2h. Wells were washed three times with wash buffer and incubated for 1hr with streptavidin conjugated to horseradish peroxidase. Wells were washed three times with wash buffer and a colorimetric reaction was initiated by adding equal parts H₂O₂ and tetramethylbenzidine. The reaction was stopped after 20 min with 2N H₂SO₄. IL-8 concentration was assessed by measuring optical density at 450nm using a microplate reader (Molecular Devices, Sunnyvale CA). Samples were compared to a standard curve provided by R&D Systems to determine IL-8 concentration using the SOFTmax PRO software (Molecular Devices, Menlo Park, CA). Values were normalized to total protein in SDS-lysed HUVECs using the Dc Bio-Rad protein assay (Hercules, CA). A standard curve was prepared with bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, MO) in 0.4% SDS.

**TF Assay**

Cellular TF levels were measured with a TF-specific ELISA (American Diagnostica, Stamford, CT). HUVECs treated in T25 flasks were removed from 37º C, media aspirated and discarded, and immediately placed on ice. Cells were mechanically lysed in 750 µl PBS with a cell scraper (Fisherbrand, Pittsburgh, PA). Lysis was repeated and a total of 1500 µl lysate was centrifuged at 4,000 rpm for 10min at 4º C. Pellets were resuspended by sonication in the presence of 1:100 dilution of each protease inhibitor and phenylmethylsulfonyl fluoride in SKL buffer (50mM HEPES, 150 nM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 1% Triton X-100, 10% glycerol). Samples were centrifuged at 14,000 rpm for 15 min at 4º C and supernatants added to 96-well plates pre-coated with murine anti-human TF antibody. After 3hr, wells were washed four times with wash buffer (0.1% Triton X-100 in PBS) and incubated with biotinylated
antibody fragments recognizing bound TF. After 1 hr, wells were washed and incubated with streptavidin conjugated horseradish peroxidase for 20 min. A colorimetric reaction was initiated with the addition of tetramethylbenzidine. This reaction was stopped after 20 min with 0.5 N H$_2$SO$_4$. TF concentration was assessed by measuring optical density at 450 nm using a microplate reader (Molecular Devices). Samples were compared to a TF standard curve provided by American Diagnostica using the SOFTmax PRO (Molecular Devices) software. Values were normalized to total cell protein measured with the Dc Protein Assay from Bio-Rad. A standard curve was prepared with BSA (Sigma Aldrich) in SKL buffer.

**Real-Time PCR Analysis**

Total cellular RNA was initially extracted from duplicate flasks for each condition studied and purified with an RNAeasy minikit (Qiagen Inc., Valencia, CA). Five μg of RNA was reverse transcribed to cDNA with AMV reverse transcriptase (Invitrogen, San Diego, CA). A quantitative standard curve ranging from 500 pg to 250 ng of cDNA was created with the Roche Light Cycler (Roche, Indianapolis, IN) by monitoring the increasing fluorescence of PCR products during amplification. Once the standard curve was established, quantitation of unknowns was determined and adjusted to the expression of 18S RNA. Melting curve analysis was conducted to determine the specificity of the amplified products and to ensure the absence of primer-dimer formation. All products obtained yielded the correct melting temperature. Electrophoresis of amplified products revealed single products of the expected size for IL-8, and TF, and 18S. In addition, DNA sequencing verified their identity. The following primers were synthesized and gel-purified at the Yale DNA Synthesis Laboratory, Critical Technologies:
IL-8        forward 5’-GACAAGAGCCAGGAAGAAAC-3’ (459 bp)  
            reverse 5’-CTACAACAGACCCACAATAAC-3’

TF         forward 5’-GAAGCAGACGTACTTGGCACGG-3’ (121 bp)  
            reverse 5’-CCGAGGTTTGTCTCCAGGTA-3’

18S        forward 5’-GATATGCTCATGTGCTTG-3’ (236 bp)  
            reverse 5’-AATCTTCTTCAGTGCCTCCA-3’

*Statistical Analysis*

All results are reported as a mean ± standard error. Data was analyzed with one-way ANOVA using the SigmaStat software (Jandel Scientific, San Rafael, CA). If normality failed, Kruskal-Wallis one-way analysis of variance on ranks was performed. A value of P < 0.05 was considered significant. IL-8 ELISA results are given as the mean ± SE of triplicate determinations from an experiment representing 3 identically conducted ones.

*All of the above protocols were executed entirely by the student.*
RESULTS

*Regulation of IL-8 protein in HUVECs by LPS, PIC, PG, DEX, and MT*

In the first series of experiments, HUVECs were incubated for 24 and 48h without (control) or with LPS (1µg/ml), PIC (1µg/ml), or PG (1µg/ml) in serum-free or stripped serum conditions. IL-8 concentrations in culture medium were determined by ELISA and normalized to cell protein. Under serum-free conditions, LPS increased IL-8 levels 29- and 6-fold over control at 24h (0.23 ± 0.23 vs. 0.68 ± 0.77 pg/µg protein, p > 0.05) and 48h (1.54 ± 0.70 vs. 9.78 ± 2.48 pg/µg protein, p > 0.05) respectively (Figure 1A). Incubation with LPS in stripped serum also yielded 7- and 5-fold increases in IL-8 over control at 24h (2.75 ± 0.27 vs. 20.53 ± 3.30 pg/µg protein, p > 0.05) and 48h (8.17 ± 0.87 vs. 42.19 ± 5.78 pg/µg protein, p > 0.05), respectively (Figure 1B). In the PIC treatment group, we observed a significant induction of IL-8 expression over control, LPS, and PG treatment groups (*p < 0.05). Under serum-free conditions, PIC elicited a 1050- and 47-fold increase over 24h (0.23 ± 0.23 vs. 245.92 ± 69.48 pg/µg protein, p < 0.05) and 48h (1.54 ± 0.70 vs. 72.94 ± 54.89 pg/µg protein, p < 0.05), respectively, above IL-8 levels in the control group. Similarly, IL-8 levels in PIC-treated cells increased 196- and 199-fold at 24h (2.75 ± 0.27 vs. 541.18 ± 114.90 pg/µg protein, p < 0.05) and 48h (8.17 ± 0.87 vs. 1628.90 ± 277.09 pg/µg protein, p < 0.05), respectively, in stripped serum. Although PG treated HUVECs in serum-free conditions showed 16- and 2.5 fold increases in IL-8 expression, no change in IL-8 levels were appreciated in stripped serum. These results indicated that PIC treatment had the most profound effect on IL-8 levels in HUVECs. We also note that IL-8 expression is increased in all groups when treatments were performed in stripped serum versus serum free conditions. Trace immunoglobulins,
Figure 1. Regulation of IL-8 levels in HUVECs by LPS, PIC, and PG. HUVECs were incubated for 24 and 48h without (control) or with LPS (1µg/ml), PIC (1µg/ml), or PG (1µg/ml) in (A) serum free medium, and (B) stripped serum. IL-8 concentrations in culture medium were determined by ELISA and normalized to cell protein. * $p < 0.05$. Data shown are mean ± SE of three separate experiments conducted in triplicate (n=3).
albumins, growth stimulating molecules, and other serum factors may contribute to more robust gene and protein expression in cells treated with stripped serum versus serum free media.

In a second series of experiments, HUVECs in stripped serum were maintained for 48h without (control) or with LPS (1µg/ml), PIC (1µg/ml), PG (1µg/ml) and also with DEX (100nM) and MT (10 µM). For combined DEX and MT treatments, 1nM and 0.5 µM, respectively, were used. Treatment of HUVECs with LPS and PIC elicited 4- and 62-fold increases, respectively, in IL-8 levels over control whereas PG treatment had no effect (Figure 2). In LPS-treated HUVECs, DEX treatment reduced IL-8 levels by over 60% (20.6 ± 5.22 vs. 7.9 ± 0.26 pg/µg protein, p > 0.05). MT treatment, whether used alone or in combination with DEX, had no effect on the LPS-mediated increases in IL-8 expression in HUVECs (Figure 2). Incubation of HUVECs with DEX decreased IL-8 expression in PIC-treated cells by over 90% (334.66 ± 59.851 vs. 25.87 ± 4.63 pg/µg protein, p > 0.05). In contrast, incubation with MT induced a 2.5-fold increase in IL-8 expression over PIC treatments (334.66 ± 59.851 vs. 808.46 ± 107.96 pg/µg protein, p > 0.05). Incubation of HUVECs with both DEX and MT showed no change in IL-8 levels in PIC-treated cells. In PG-treated cells, incubation with DEX, MT, or both did not produce observable change in IL-8 levels. This indicated that DEX may have anti-inflammatory properties in a therapeutic setting. These results do not support an anti-inflammatory role for MT especially given the enhanced IL-8 expression. Combined DEX and MT treatment did not provide any significant anti-inflammatory action. We note that IL-8 expression in the PIC-treated (Figure 2) group is less robust than was
Figure 2. Regulation of IL-8 levels in HUVECs by LPS, PIC, PG, DEX, and MT. HUVECs were incubated for 48h without (control) or with LPS (1µg/ml), PIC (1µg/ml), or PG (1µg/ml) in stripped serum. Treatments with DEX (100nM), MT (10nM), or DEX (1nM) and MT (0.5nM) were simultaneously performed. IL-8 concentrations in culture medium were determined by ELISA and normalized to cell protein. Data shown are mean ± SE of a single experiment conducted in triplicate (n=1).

Figure 3. Effect of LPS, PIC, and PG on IL-8 mRNA expression in HUVECs. HUVECs were maintained for 24h in stripped serum without (control) or with LPS (1µg/ml), PIC (1µg/ml), or PG (1µg/ml). IL-8 mRNA expression was quantitated by real-time PCR analysis as described in the METHODS section. Data shown are mean ± SE three separate experiments (n=3).
observed in a previous experiment also conducted in stripped serum (Figure 1B). The HUVECs in the experiment presented in Figure 2 were of the higher passage than the HUVECs used in Figure 1B. This may contribute to less robust cellular machinery especially when stressed with microbial products.

**Effect of LPS, PIC, and PG on IL-8 mRNA expression in HUVECs**

HUVECs were maintained for 24h in stripped serum without (control) or with LPS (1µg/ml), PIC (1µg/ml), or PG (1µg/ml). Expression of IL-8 mRNA was quantitated by real-time PCR analysis as described in the Methods Section. LPS treatment of HUVECs elicited an 11-fold increase in IL-8 mRNA expression over control (0.39 ± 0.17 vs. 4.32 ± 3.31 IL-8 mRNA/18S, p > 0.05) (Figure 3). HUVECs treated with PIC showed a marked 450-fold increase in IL-8 mRNA expression over control (0.39 ± 0.17 vs. 177.99 ± 50.442 IL-8 mRNA/18S, p > 0.05). We observed no change in IL-8 mRNA expression by HUVECs incubated with PG. This indicated that PIC most dramatically upregulated IL-8 mRNA expression, a pattern congruent with the observed PIC-enhanced IL-8 protein expression.

**Regulation of TF protein in HUVECs by LPS, PIC, PG, DEX, and MT**

HUVECs were incubated for 24 and 48h without (control) or with LPS (1µg/ml), PIC (1µg/ml), or PG (1µg/ml) in stripped serum. TF concentrations in culture medium were determined by ELISA and normalized to cell protein. We observed that LPS treatment promoted no change in the level of TF expression compared to control (Figure 4). In contrast, treatment with PIC elicited approximately a 60-fold increase in TF levels over control (0.05 ± 0.05 vs. 3.32 ± 0.15 pg/µg protein, p > 0.05). HUVECs treated with PG showed no change in TF expression compared control. In a separate experiment, HUVECs in stripped serum were maintained for 48h without (control) or with LPS
Figure 4. Regulation of TF levels in HUVECs by LPS, PIC, and PG. HUVECs were incubated for 24 and 48h without (Control) or with LPS (1µg/ml), PIC (1µg/ml), or PG (1µg/ml) in stripped serum. TF concentrations in culture medium were determined by ELISA and normalized to cell protein. Data shown are mean ± SE from three separate experiments conducted in duplicate (n=3).

Figure 5. Regulation of TF levels in HUVECs by LPS, PIC, PG, DEX, and MT. HUVECs were incubated for 48h without (control) or with LPS (1µg/ml), PIC (1µg/ml), or PG (1µg/ml) in stripped serum. Treatments with DEX (100nM), MT (10nM) were simultaneously performed. TF concentrations in culture medium were determined by ELISA and normalized to cell protein. Data shown are raw values (n=1).
(1µg/ml), PIC (1µg/ml), PG (1µg/ml), DEX (100nM) and MT (10 µM). We observed that LPS treatment did not affect levels of TF (Figure 5). In addition, DEX or MT did affect TF expression in cells treated with LPS. We observed a 200-fold increase in TF levels in PIC-treated cells when compared to control (0.01 vs. 1.86 pg/µg protein). An additional 2.5-fold increase in TF expression was noted in PIC-treated HUVECs incubated with DEX (1.86 vs. 4.9 pg/µg protein). MT treatment had no effect on TF levels in cells treated with PIC. We also noted that PG treatment did not affect TF levels compared to control. There was no observable effect of DEX or MT treatment on TF levels in cells treated with PG. These results suggest that PIC elicited the most profound increase in TF expression. DEX and MT, however, are most likely not effective treatments for mitigation of TF expression and subsequent prevention of thrombosis.
DISCUSSION

Cerebral palsy includes an array of sensorineural and motor deficits affecting 2-3/1000 neonates (1). Although the pathogenesis of CP is currently debated, it is likely the result of fetal neural compromise in the antepartum period. There are several observations that have been made regarding the placentas of neonates affected by CP which may offer clues as to the mechanism of CP incidence. These include fetal thrombotic vasculopathy, chronic villitis with obliterative fetal vasculopathy, and chorioamnionitis with fetal vasculitis (3, 4). The connection between these thrombo-inflammatory findings and CP may involve infections in the large fetoplacental vessels and the subsequent inflammatory response and thrombosis. The umbilical vessels are in direct continuity with fetal capillary beds, most importantly the CNS (3). The umbilical vessels can therefore serve as a conduit for microbes, inflammatory molecules, and pro-thrombotic molecules to pass directly into the fetal circulation. In the setting of chronic villitis, local infection induces an inflammatory response recruiting neutrophils and other inflammatory cells to the site of insult (5). If uncontrolled, this inflammatory process may be extended beyond the local placental vasculature and via umbilical vessels. Activated immune cells and endothelial cells secrete proinflammatory cytokines such as IL-6, IL-8, IL-12, and TNF-α which may reach downstream fetal tissues (6,8). Although the exact mechanisms are still unknown, the presence of these mediators in fetal circulatory beds, such as the developing CNS, may cause inflammatory changes in the local vasculature and tissue ischemia. Associated with the inflammatory response is the expression of pro-thrombotic molecules such as TF by the endothelium (21). In the case
of thrombotic vasculopathy and obliterative fetal vasculopathy where endothelial cell damage is noted, platelet activation and thrombin secretion may extend beyond the local placental vasculature and access downstream fetal tissues (5,7). The current study focuses on microbe induced changes occurring in the endothelium of fetal placental vasculature. We have a potential therapeutic model that, on the cellular level, may blunt an inappropriately robust inflammatory and thrombotic response to microbial pathogens.

Microbial pathogens are capable of eliciting host immune responses via specific signaling receptors. The TLR family includes receptors which specifically interact with bacterial and viral products and the presence of TLRs in placental tissue has been demonstrated in previous studies (16, 17). In this study we show that ligands for TLR-2, -3, and -4 differentially modulate inflammatory cytokine expression in HUVECs. We note a profound increase in both IL-8 mRNA and protein expression in cells treated with PIC. Our data also show a moderate increase in IL-8 mRNA and protein in LPS treated cells, a finding reported in prior studies (17). These results show that HUVECs are exquisitely sensitive to PIC, a constituent of viruses and a TLR-3 ligand, and suggests an important role for viral infection in umbilical inflammation. The observed profound induction of IL-8 may be a significant contributing factor in the inflammatory placental lesions association with CP (4, 7).

This study also explores the role of TLR mediated signaling pathways in thrombosis. We report an increase in TF expression in PIC-treated cells, again implying a TLR-3 specific pathway. We conclude that the TLR-3 mediated signaling pathway enhances both inflammatory and thrombotic responses in HUVECs. These findings agree with the observation that inflammatory cytokines are correlated with an increase
the expression of pro-thrombotic factors, namely TF (19-21). This association may be critical in the evolution of concurrent villitis with obliterative vasculopathy seen in the placentas of fetuses with resulting CP (7). The implication of viral products in both inflammation and thrombosis imparts significance on the management of evolving CP-associated lesions. A viral infection in the antepartum period may not declare itself until the resulting placental lesions are observed.

We additionally explore the roles of GC and MT in large vessel inflammation and thrombosis. Our data show a marked decrease in IL-8 production in PIC-treated HUVECs also incubated with DEX. This effect agrees with prior studies demonstrating the anti-inflammatory properties of DEX in placental tissue (25). These observations suggest a mechanism for GC to interact with TLR pathway-associated molecules to reduce IL-8 and potentially other inflammatory molecules. This interaction may or may not occur at the level of NF-κB activity, a transcription factor shown to be regulated by both TLR and GC receptor signaling (14, 22). Although preliminary, our data also show a potential anti-thrombotic effect of GC in HUVECs stimulated with PIC. We conclude that GC may offer therapeutic anti-inflammatory and anti-thrombotic action in placental vessels.

MT, in contrast, mediated an increase in IL-8 expression in PIC-treated cells and preliminary data showed no mitigation of TF expression. The observed immunostimulatory effects on HUVECs may be attributed to a similar stimulation of IL-2, IL-6, and IL-12 cytokine expression noted in hematopoietic cells by Maestroni et al (30). There is little existing data on the effects of MT on endothelial cell inflammatory cytokine expression. We recognize that there may dose-dependent variation in MT
activities which may include concentrations that are pro-inflammatory versus anti-inflammatory. This area of study may be expanded to include MT concentrations both lower and higher than 10µM. Based on the current findings, MT is not a favorable anti-inflammatory or anti-thrombotic agent in placental tissues.

There are inherent strengths and weaknesses in the current study related to research design, data collection, and analysis. We feel that the current literature strongly supports the rationale for studying the role of glucocorticoids and melatonin in inflammation. These substances are currently used in human therapeutic regimens and GCs are readily used in pregnancy for various indications. Regarding HUVECs as a model system, the cells are readily available and reasonably easy to manipulate. As discussed previously, a single cell population without associated vascular accessory cells would not allow observation of changes in vascular integrity that may be occurring with inflammatory and thrombotic changes. While 4 x 10^5 cells were needed per condition for IL-8 assays, the yield for HUVEC TF expression is much lower and 1 x 10^6 or more cells per treatment was required. This reduced the relative number of TF experiments that were conducted in the given period of time. We plan to expand the numbers for all experiments, therefore improving statistical analysis of data using ANOVA. Finally, this study addresses the mechanisms for the development of fetoplacental vascular lesions. Although we associate these lesions with CP based on findings in the literature, a causative relationship has yet to be elucidated. In future studies, the roles of inflammatory cytokines could be explored in fetal vascular models, and if possible, a fetal neurovascular model.
We conclude that treatment with PIC, and to a lesser extent LPS, promotes inflammatory cytokine production in HUVECs. PIC treatment also enhanced TF expression. These findings suggest that viral products acting through the TLR-3 specific signaling pathway enhance pro-inflammatory and pro-thrombotic responses in feto-placental vasculature. We also show that DEX treatment suppresses PIC effects on cytokine production in HUVECs and, preliminarily, also suppresses TF expression. The mechanism whereby DEX interacts with the TLR signaling pathway may involve NF-κB. These interactions demonstrate a potential role for GC in prevention of inflammatory and thrombotic events which may contribute to placental pathology associated with CP.
REFERENCES


