Pentoxifylline Inhibits Tumor Necrosis Factor-Alpha Induced Synthesis of Complement Component C3 in Human Endothelial Cells

Eric Brian Hoie,† Timothy Robert McGuire,* Patricia Mary Leusch, and Terrence Leo Zach

†College of Pharmacy, University of Nebraska Medical Center; and ‡Medicine, University of Nebraska Medical Center; 986045 Nebraska Medical Center, Omaha, NE 68198-6045, U.S.A. Received March 15, 2004; accepted July 8, 2004

Vascular endothelium is a major target for the inflammatory damage that occurs with multiple organ dysfunction associated with sepsis and other trauma. The growing appreciation of endothelium as a target of inflammation has obscured the importance of these cells as a source of inflammatory mediators. In the following study we evaluated the ability of tumor necrosis factor-alpha (TNF) to induce the synthesis of complement component C3 in human umbilical vein endothelial cells (HUVEC) and whether pentoxifylline (PTX) could reduce C3 expression. Confluent monolayers of HUVEC were treated with increasing concentrations of TNF with and without two concentrations of PTX. Concentrations of C3 were determined every 48 h for 144 h in cellular supernatants and C3 mRNA was amplified using RT-PCR. TNF increased C3 release from HUVEC in a concentration dependent manner. PTX added at the same time as TNF significantly reduced C3 release at the 96 h time point. Consistent with data on C3 release PTX inhibited the increased C3 mRNA expression associated with TNF treatment. TFN increases C3 synthesis and release from endothelial cells which were inhibited by clinical concentrations of PTX. This data further supports the potential benefit of PTX in multiple organ dysfunction and other inflammatory processes involving the endothelium by inhibiting one of the major mediators of vascular damage.

Key words complement; C3; human umbilical vein endothelial cell (HUVEC); tumor necrosis factor (TNF)-α; pentoxifylline

The third component of complement, C3, is the keystone of the complement cascade located at the site of convergence of the classical and alternative pathways of complement activation. Products of complement activation are integral components of the immune system. Opsonization of bacteria and direct cell lysis caused by the membrane attack complex (MAC) are two of the important functions of the complement cascade. Complement activation must be balanced to provide activity against invading organisms while limiting damage against host cells. Activation of the complement cascade occurring at local sites of tissue injury can lead to formation of the MAC as well as anaphylatoxins C5a and C3a which recruit neutrophils to sites of inflammation and tissue damage.

Although the hepatocyte is the principle source of circulating C3, extrahepatic sources of C3 such as pulmonary epithelial cells, fibroblasts, and alveolar macrophages may be important at local sites of inflammation. Human endothelial cells synthesize and secrete C3 which is part of the inflammatory response of endothelial cells. Cytokines such as tumor necrosis factor-alpha (TNF) and interleukin-1 have been reported to increase C3 synthesis by endothelial cells.

TNF is a central mediator in the progression of shock following gram negative sepsis. Infusion of TNF into animals has been shown to produce vascular leakage similar to that observed following Escherichia coli sepsis and passive immunization of baboons with monoclonal anti-TNF prevented death after a lethal dose of E. coli. Lipopolysaccharide (LPS) derived from gram negative bacteria stimulates alveolar macrophages to produce TNF. TNF triggers a number of events that contribute to the pathogenesis of inflammatory disorders. TNF increases neutrophil adherence to endothelial cells, augments neutrophil oxygen radical production, and alters endothelial permeability.

Pentoxifylline (PTX) has been shown to prevent TNF-induced lung injury in guinea pigs. TNF treated animals experienced an increase in albumin permeability and an accumulation of leukocytes in alveolar spaces compared to controls. TNF-PTX treated animals showed no increase in albumin permeability or alveolar leukocyte counts. Similar results have also been obtained following injection of E. coli or endotoxin to dogs and guinea pigs. In both cases PTX treated animals did not experience lung injury consistent with endothelial damage. PTX has also been shown to improve cardiovascular function in adults with septic shock and improve survival in premature infants with sepsis. While PTX is known to decrease TNF secretion from neutrophils we have demonstrated for the first time that PTX antagonizes the effect of TNF on C3 synthesis in endothelial cells which may explain in part it’s protection against vascular damage during sepsis.

METHODS

Human Umbilical Vein Endothelial Cell Cultures

HUVEC Unless specified otherwise all reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Endothelial cells were isolated from human umbilical veins using a modification of a method previously described. Umbilical cords were obtained following normal delivery, the vein was washed with phosphate buffered saline (PBS) to remove blood, and the luminal surfaces were treated with collagenase (1 mg/ml) for 10 min to detach endothelial cells. The cells were removed by flushing the veins with media 199 (M199) and pelleted by centrifugation. The cells were resuspended in M199 supplemented with fetal bovine serum (FBS) 20%, endothelial cell growth factor (ECGF) 50 µg/ml, heparin sodium 100 U/ml, penicillin 100 U/ml, and streptomycin 100 µg/ml. The cells were plated in 25 cm² tissue culture flasks and incubated at 1 atmosphere of humidified 5% CO₂ at 37°C. Confluent monolayers were detached with trypsin EDTA 0.05% and plated in 75 cm² tissue culture
flasks. Confluent monolayers from the 75 cm² flasks were detached as above and plated into 24-well tissue culture plates for TNF and PTX dose and time response studies. The effect of TNF and PTX combinations on C3 release from HUVEC was measured using an immunoassay.

Primary endothelial cells (BioWhittaker, Walkersville, MD, U.S.A.) were used for analysis of C3 mRNA. The endothelial cells were initially grown to confluence in 25 cm² tissue culture flasks using endothelial growth media 2 (EGM-2, BioWhittaker) and then trypsinized and plated into 75 cm² tissue culture flasks to determine the effects of TNF and PTX on C3 mRNA.

**C3 Determination by ELISA** C3 concentrations were measured in duplicate in HUVEC supernatant using a modification of the method previously described by Zach et al. Briefly, 96-well microtiter plates were coated with 0.1 ml of a 1:400 dilution of goat antihuman C3 (Atlantic Antibodies) in carbonate/bicarbonate buffer (pH 9.6), incubated overnight at 4 °C, and washed 3 times with 0.05% Tween 20 in PBS. Cell culture supernatant samples (0.1 ml) were added to duplicate wells and incubated for 1 h at 37 °C. After 3 washes, 0.1 ml of a 1:800 dilution of peroxidase-conjugated goat antihuman C3 (Cappel Laboratories) was added to each well and incubated at 37 °C for 1 h. The developing reagent consists of 0.4 mg/ml of o-phenylenediamine in 0.2 M Na₃HPO₄ (pH 5.0) containing 0.4 μl 35% H₂O₂ per ml. Color was developed for 25 min and absorbance measured at 492 nm. Purified human C3 was used for the standard curve and the assay validated using radial immunodiffusion. The ELISA is specific for human C3 with no cross-reactivity with the fetal bovine serum used to supplement the growth media.

**RNA Isolation, cDNA Synthesis, and Polymerase Chain Reaction Amplification** Total RNA was isolated from HUVEC with RNA-Stat 60 (Tel-Test Inc., Friendswood, TX) according to the manufacturers instructions. The RNA pellet was resuspended in 50 μl of 1:400 dilution of goat antihuman C3 (Atlantic Antibodies) in carbonate/bicarbonate buffer (pH 9.6), incubated overnight at 4 °C, and washed 3 times with 0.05% Tween 20 in PBS. Cell culture supernatant samples (0.1 ml) were added to duplicate wells and incubated for 1 h at 37 °C. After 3 washes, 0.1 ml of a 1:800 dilution of peroxidase-conjugated goat antihuman C3 (Cappel Laboratories) was added to each well and incubated at 37 °C for 1 h. The developing reagent consists of 0.4 mg/ml of o-phenylenediamine in 0.2 M Na₃HPO₄ (pH 5.0) containing 0.4 μl 35% H₂O₂ per ml. Color was developed for 25 min and absorbance measured at 492 nm. Purified human C3 was used for the standard curve and the assay validated using radial immunodiffusion. The ELISA is specific for human C3 with no cross-reactivity with the fetal bovine serum used to supplement the growth media.

**RESULTS**

C3 concentrations were minimal in both control and treatment wells at 48 h. At 96 and 144 h TNF treatment produced a dose and time–response effect on C3 production (Table 1). No C3 protein was detected in wells not treated with TNF. There was no direct evidence of cell-kill caused by TNF. Cell counts were slightly lower in TNF treated cells, however, this appeared to be a result of a change in the cell morphology as the TNF treated cells were elongated compared to the controls. PTX (3, 30 mcg/ml) significantly decreased C3 concentrations in TNF treated wells at 96 h (p=0.0016) (Table 2). The ability of PTX to inhibit C3 protein production at 144 h strongly trended towards significance (p=0.07) (Table 3).

C3 mRNA is expressed in HUVEC controls. There was approximately a 50% increase in intensity seen with TNF treatment and PTX inhibited the effects of TNF on C3 mRNA. The C3/beta-actin ratios for the four conditions until assayed. Identically treated plates were used for determination of cell counts at each of the sampling times and C3 concentrations were corrected for cell count.

**Effects of Pentoxifylline on TNF Treated HUVEC**

PTX was diluted in HUVEC growth media and added to confluent monolayers of HUVEC at concentrations of 3 and 30 μg/ml. Two hours after PTX addition, TNF, 10 or 30 ng/ml, was added to each of the PTX treated wells. Supernatants were collected and stored at −70 °C every 48 h for 144 h and C3 concentrations were corrected for cell count.

**Effects of Pentoxifylline and TNF on C3 mRNA** Confluent HUVEC monolayers in 75 cm² flasks were treated with PTX, 30 μg/ml, for 2 h. TNF, at a concentration of 30 ng/ml, was added to both the flask receiving PTX pre-treatment and the flask containing media alone. Two additional flasks, one without either PTX or TNF, and one with only PTX were also included. Treatment continued for 96 h at which time the total RNA was collected as previously described.

**Statistical Analysis** C3 concentrations were reported as mean±S.D. TNF and PTX effects on C3 release from HUVEC were compared by one-way analysis of variance.

<table>
<thead>
<tr>
<th>TNF (ng/ml)</th>
<th>C3 at 96 h (ng/ml)</th>
<th>C3 at 144 h (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.13 ± 0.15</td>
</tr>
<tr>
<td>0.1</td>
<td>0</td>
<td>0.49 ± 0.71</td>
</tr>
<tr>
<td>1.0</td>
<td>2.2 ± 3.4</td>
<td>4.2 ± 6.0</td>
</tr>
<tr>
<td>10</td>
<td>13.4 ± 6.6</td>
<td>22.5 ± 14.6</td>
</tr>
<tr>
<td>30</td>
<td>19.7 ± 6.2</td>
<td>27.9 ± 9.4</td>
</tr>
</tbody>
</table>

a) Represent the mean plus or minus standard deviation of 6 replicates.

<table>
<thead>
<tr>
<th>TNF (ng/ml)</th>
<th>TNF alone</th>
<th>PTX: 3 μg/ml ± S.D. (%)*</th>
<th>PTX: 30 μg/ml (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.13 ± 0.15</td>
<td>0.71</td>
</tr>
<tr>
<td>10</td>
<td>13.4 ± 6.6</td>
<td>5.86 ± 3.74 (43.6)</td>
<td>6.31 ± 3.63 (47)</td>
</tr>
<tr>
<td>30</td>
<td>19.7 ± 6.2</td>
<td>8.88 ± 3.83 (45.0)</td>
<td>7.47 ± 3.53 (38)</td>
</tr>
</tbody>
</table>

a) Average of 6 wells plus or minus standard deviations. b) Percentage of TNF alone values. p=0.0016, ANOVA.
were; media alone controls (0.26), PTX alone (0.17), TNF alone (0.39), and PTX/TNF combination (0.22) (Fig. 1).

**DISCUSSION**

The expression of C3 mRNA without corresponding increased protein secretion, which we reported in these experiments, is consistent with prior reports on the constitutive expression of the C3 gene and the intracellular storage of C3 protein. The release of C3 is stimulated by various inflammatory molecules such as TNF.17,18

In the following investigation we demonstrated that HUVEC treated with TNF had an increased expression of C3 mRNA and an increased release of C3 protein. PTX was able to inhibit both the expression of C3 mRNA and the release of C3 protein. While the mechanism of PTX mediated reduction in C3 expression is not known, the effect of PTX on TNF mediated inflammation has been studied. Recently, PTX has been reported to inhibit the expression of an inflammatory chemokine (fractalkine) after treatment of vascular smooth muscle cells with TNF. This effect may have been the result of inhibition of nuclear factor kappa-B (NFκB), a transcriptional regulator involved in the gene expression of many inflammatory molecules, including complement.19 It remains an open question whether the effect of PTX on TNF mediated C3 production is a result of inhibiting NFκB.

It has been previously reported that TNF stimulates C3 synthesis by HUVEC but there are no reports on the use of available therapeutic agents to inhibit this effect. While TNF and complement are but two of the numerous pro-inflammatory mediators their roles in endothelial dysfunction have been well established. Selective blockade of inflammatory cytokines has met with limited success in studies of sepsis and septic shock. Multi-drug therapy directed at specific pro-inflammatory mediators may be necessary in the treatment of conditions such as septic shock, ARDS, myocardial infarction and stroke.

Complement activation is well documented in ARDS20—23 and sepsis24—26 and has also been implicated in tissue necrosis following myocardial infarction and stroke.27,28 Activation of the complement cascade at the site of injury leads to infiltration of neutrophils and subsequent tissue damage. Individuals with ARDS have elevated plasma levels of C3 and C5a,21 C5a,22 and soluble products of the terminal complement complex (C5b—9)23 indicating activation of the complement cascade.

Complement activation has also been reported to correlate with outcomes in individuals with sepsis and shock. In two separate studies of complement activation and sepsis, significantly elevated levels of complement were reported in septic patients with a further increase in patients with shock and in patients that subsequently died.24,25

Activation of complement has been demonstrated in a rat model of myocardial infarction.27 Inhibition of complement activation led to a significant reduction in myocardial necrosis following reperfusion. Similarly, activation of the complement system was demonstrated in mice following occlusion of the middle cerebral artery followed by reperfusion.28 The sections of the brain affected by stroke and reperfusion showed increased expression of complement while areas of the brain that were unaffected showed little or no complement activation. Administration of a complement antagonist prior to the stroke caused a modest reduction in cerebral injury.

The role of C3 in many disease processes is well established and briefly described above. The benefit of PTX in various diseases is more ambiguous. However it has been reported to have value in many inflammatory and vascular pathologies including, multiple organ dysfunction, acute renal failure, claudication, stroke and ischemic cardiomyopathy.29 How much of the value of PTX in these conditions is a result of C3 antagonism is unknown but is plausible given the important role of C3 and the ability of PTX to reduce C3 synthesis as reported in this study.

**CONCLUSION**

We have demonstrated that PTX antagonizes the effect of TNF on C3 synthesis in human endothelial cells. The case that PTX may be beneficial as part of a treatment regimen in severe pro-inflammatory conditions where endothelium is a target is strengthened by the results reported in this study. Future clinical investigations should include evaluation of molecular markers for inflammation and tissue damage to better describe pentoxifylline mechanism of action.

**Acknowledgments** This work was supported in part by grants from The University of Nebraska Medical Center, the American Association of Colleges of Pharmacy and the American College of Clinical Pharmacy.

**REFERENCES**