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P-cresol, a uremic toxin, decreases endothelial cell response to inflammatory cytokines

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P-cresol, a uremic toxin, decreases endothelial cell response to inflammatory cytokines.

Background. Infectious diseases are among the most morbid events in uremia. The uremic toxin p-cresol may play a role in the immunodeficiency of uremia by depressing phagocyte functional capacity. Leukocyte adhesion to endothelium, a key event in the immune response, is mediated by endothelial adhesion molecules. These include intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, which are induced by various inflammatory cytokines. We asked whether p-cresol alters endothelial adhesion molecule expression and modifies endothelial/leukocyte adhesion.

Methods. Human umbilical vein endothelial cells (HUVEC) were incubated with p-cresol in the presence or absence of tumor necrosis factor (TNF) or interleukin-1β (IL-1β). Thereafter, the endothelial molecules ICAM-1, VCAM-1, and E-selectin were quantitated and the monocyte (THP-1) adhesion to HUVEC measured.

Results. P-cresol decreased cytokine-induced protein and mRNA expression of ICAM-1 and VCAM-1. In addition, p-cresol significantly decreased the adhesion of THP-1 to cytokine-stimulated HUVEC.

Conclusions. P-cresol may play a role in the immune defect of uremic patients by inhibiting cytokine-induced endothelial adhesion molecule expression and endothelium/monocyte adhesion.

Uremic patients often display an immunodeficiency state, and infection remains one of the major causes of mortality in patients with chronic renal failure (CRF) [1]. Studies strongly suggest that uremic toxins are involved in the impaired immune response of CRF patients. For example, methylguanidine significantly inhibits tumor necrosis factor (TNF) production in vitro and in vivo [2], parathyroid hormone (PTH) inhibits B cell proliferation [3], and polyamines decrease TNF-induced gene expression in endothelial cells [4].

It has been suggested that the uremic toxin p-cresol (4-methylphenol, molecular weight 108.1 D) plays a role in the immunodeficiency of uremia because it depresses phagocyte functional capacity [5] and inhibits the release of platelet-activating factor (PAF) by macrophages [6]. P-cresol is an end product of protein catabolism. It is a partially lipophilic moiety, which strongly binds to plasma proteins under normal conditions. A part of the unconjugated p-cresol is removed via urine. Furthermore, in uremia, modifications in the intestinal flora result in the specific overgrowth of bacteria that are p-cresol producers [7]. Plasma concentrations of total p-cresol increase progressively during the development of chronic renal failure. In uremic patients, serum levels of p-cresol are increased about tenfold and those of free non-protein-bound p-cresol are even higher [8].

Leukocyte adhesion to endothelium is a key event in immune response, since leukocytes have to cross the endothelial barrier to migrate to the sites of infections. Adhesion molecules of both cell types mediate leukocyte adhesion to endothelium. Such adhesion molecules include E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells. E-selectin, a member of the selectin family, is not expressed by resting endothelium. After endothelial stimulation by inflammatory cytokines or lipopolysaccharides (LPS), E-selectin is strongly and rapidly induced on endothelial surface and thus mediates leukocyte rolling on inflamed endothelium. ICAM-1 and VCAM-1 are two members of the immunoglobulin gene superfamily. They are involved in firm adhesion of leukocytes to endothelium. ICAM-1 is constitutively expressed by resting endothelial cells and its expression is strongly enhanced by cytokines. ICAM-1 binds leukocyte integrins LFA-1 and Mac-1 and mediates adhesion of neutrophils, lymphocytes, and monocytes. VCAM-1,

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weakly expressed by resting endothelium, is strongly up-regulated by inflammatory stimuli. VCAM-1 is a ligand for VLA-4, an adhesion molecule expressed on lymphocytes, monocytes, and eosinophils [9].

To our knowledge, no studies have investigated the effect of p-cresol on endothelial adhesion molecules. Since leukocyte adhesion to endothelium plays a key role in immune response and inflammation, we asked whether p-cresol altered endothelial adhesion molecule expression and modified endothelial/leukocyte adhesion.

METHODS

Reagents and antibodies

P-cresol was obtained from Supelco (Sigma-Aldrich, Saint Quentin Fallavier, France). Bovine serum albumin (BSA) and sodium azide were purchased from Sigma-Aldrich and methanol from Carlo Erba (Milano, Italy). Calcein-AM was obtained from Molecular Probes (Eugene, OR, USA), TNF from Tebu (Le Perray en Yvelines, France), and interleukin-1β (IL-1β) from Boehringer Mannheim Biochemica (Meylan, France). Monoclonal antibodies against VCAM-1 (clone 1G11) and E-selectin (clone 12B6), and irrelevant control IgG were purchased from Immunotech (Marseille, France). Monoclonal antibody (mAb) against ICAM-1 (clone F431C2) was obtained from Biocytex (Marseille, France). FITC-conjugated sheep anti-mouse Ig $F(ab')_2$ fraction (Silenus) was from Eurobio (Les Ulis, France). QIFIKIT® beads were from Dako (Trappes, France).

EGM-2 medium was purchased from Clonetics Biowhittaker (Verviers, Belgium). Phosphate buffered saline (PBS), RPMI 1640 medium with glutamax-1, trypsin-EDTA solution, gelatin, Hepes buffer, and penicillin-streptomycin were obtained from Life Technologies (Cergy-Pontoise, France). Fetal bovine serum (FBS) was from Dominique Dutscher (Brumath, France). Human serum albumin (HSA) solution 20% was obtained from LFB (Courtaboeuf, France).

RNeasy® Mini Kit was obtained from Qiagen (Courtaboeuf, France) and SuperScript™ One-Step RT-PCR was from Life Technologies (Cergy-Pontoise, France). First Strand cDNA Synthesis Kit for RT-PCR and Taq DNA polymerase were purchased from Roche (Meylan, France).

Endothelial cell culture

Human umbilical vein endothelial cells (HUVEC) were recovered from umbilical cord vein by collagenase digestion as previously described [10]. Cells were seeded on gelatin-coated culture flasks and grown in EGM-2 medium under standard cell culture conditions (humidified atmosphere, 5% CO_2, 37°C). Cells were then detached with a 0.05% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA) solution and subcultured to the second passage on gelatin-coated 6-well or 96-well culture plates.

THP-1 cell culture

The THP-1 cell line was maintained in RPMI 1640 medium with glutamax-1 supplemented with 10% FBS, 25 mmol/L Hepes, 100 U/mL penicillin, and 100 μg/mL streptomycin, under standard cell culture conditions.

Immunofluorescence assay and flow cytometry analysis of cell adhesion molecule expression

P-cresol was diluted from a stock solution at 10 mg/mL prepared in methanol. Endothelial cells were incubated for 4 or 24 hours in EGM-2 medium containing methanol 1% (control medium) or different concentrations of p-cresol in the presence or absence of TNF (500 U/mL or 100 U/mL) or IL-1β (50 U/mL or 10 U/mL). In some experiments, human serum albumin at a final concentration of 4 g/dL was added in the medium. The concentrations of p-cresol were 100 μg/mL (10-fold uremic concentration), 10 μg/mL (uremic concentration), and 1 μg/mL (normal concentration). Endothelial cell viability, as assessed by trypan blue exclusion, was similar in the different experimental conditions and was always >85%.

After incubation, confluent HUVEC monolayers were detached with a pre-warmed 0.05% trypsin-0.02% EDTA solution for 30 seconds at 37°C. Cells were washed with PBS-0.1% BSA-0.1% sodium azide (PBS-BSA-AZ) and incubated with 50 μL of monoclonal antibodies (used at previously determined saturating conditions) for 60 minutes at 4°C. After washing with PBS-BSA-AZ, cells were labeled with 100 μL of FITC-conjugated sheep anti-mouse Ig $F(ab')_2$ fraction for 45 minutes at 4°C. After two additional washing steps, endothelial cells were analyzed using an Epics® XL flow cytometer (Beckman-Coulter, Roissy, France). Analysis was focused on the whole cell population, taken for calculation of mean fluorescence intensity. Mean fluorescence intensity was calculated by the System II™ software (Beckman-Coulter, Roissy, France) and was expressed in arbitrary units.

Quantification assay of cell adhesion molecule expression

Quantification was done with the QIFI assay (quantitative immunofluorescent indirect assay) based on the linear relationship between mean fluorescence intensity (arbitrary units) and number of binding sites of monoclonal antibodies (mAb) per cell [11]. The fluorescence intensity measured on the flow cytometer was converted into number of mAb binding sites per cell by standard beads from QIFIKIT®, including a blank preparation with no attached murine Ig together with five positive bead samples coated with increasing amounts of murine IgG. Beads were labeled with the FITC-conjugated secondary antibody. A linear regression curve was drawn

by plotting the mean fluorescence intensity measured on each bead sample and the known number of mAb binding sites per cell for each bead. The mean fluorescence intensity obtained for each antibody corrected for isotype control values was converted with the standard curve equation into the corresponding number of mAb binding sites per cell. Endothelial cells were considered positive when they expressed more than 5000 mAb binding sites per cell.

RNA extraction from HUVEC

Endothelial cells were incubated in EGM-2 medium containing methanol 1% (control), or p-cresol at 10 μg/mL or at 100 μg/mL, in the presence or absence of TNF (500 U/mL) or IL-1β (50 U/mL). After 16 hours of incubation, confluent HUVEC monolayers were detached with a pre-warmed 0.05% trypsin-0.02% EDTA solution for 30 seconds at 37°C. Cells were stored at −80°C for further RNA extraction.

Total RNA was extracted from HUVEC by an RNeasy® mini kit, and the RNA concentrations were determined by spectrophotometric measurements at 260 nm wavelength.

Two micrograms of total RNA of each sample was subjected to electrophoresis through 1% agarose gel containing formaldehyde and visualized by ethidium bromide staining.

Reverse transcriptase-polymerase chain reaction analysis of ICAM-1 and VCAM-1 mRNA

Competitive RT-PCR for ICAM-1 mRNA analysis. To analyze ICAM-1 mRNA levels by competitive reverse transcription-polymerase chain reaction (RT-PCR), as previously described [12] an internal standard was constructed that shares the same primer pair as the endogenous ICAM-1 sequences and can be distinguished by a lower size. First, the sense 1 (5′-GTCCCCCTCAAAAG TCATCCT-3′) and antisense (5′-CCGAGGTGTTCT CAAACAGCT-3′) primers were used to amplify a 581 bp ICAM-1 cDNA. An aliquot of this PCR was then amplified with the same antisense primer and a second sense 2 primer (5′-<u>AAAGTCATCCT</u>TACTGGACTC CAGAACGGGT-3′) containing in its 5-end the first 10 nucleotides of the 3′ part of the sense 1 primer (underlined in the sequence of the sense 2 primer). Finally, an aliquot of the second PCR was amplified with the sense 1 and antisense 1 to produce the internal standard, 307 bp in length. Thus, the internal standard was differentiated from the ICAM-1 cDNA sequence by size (307 vs. 581 bp) and could be amplified with the same primers. This standard was purified on a 1.5% agarose gel, and its concentration was determined by spectrophotometric measurements at 260 nm wavelength.

Total RNA (125 ng) was reverse transcribed with the First Strand cDNA Synthesis Kit for RT-PCR and antisense primer according to the manufacturer's instructions. Serial dilutions of the standard were coamplified with 2 μL of the RT product (1/10 of the RT reaction) and with the sense 1 and antisense primers. In brief, 68, 34, 17, 3.4 and 1.7 pg of ICAM-1 standard were mixed with 2 μL of the RT product in a 100 μL reaction volume containing 10 pmol of each primer, 0.2 mmol/L of each dNTP, and one unit of taq DNA polymerase. The thermal cycling reactions were denaturation at 94°C for 30 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 30 seconds (for a total of 35 cycles), followed by a termination step at 72°C for 10 minutes. The PCR products were subjected to 1.5% agarose gels, and the intensity of the ethidium bromide staining for each band was densitometrically measured with the Scion Image software (Scion Corporation, Frederick, MD, USA). The logarithm of the ratio (PCR product standard/target) was plotted versus the logarithm of the amount (pg) of the standard used in the assay. The amount of ICAM-1 cDNA produced by the RT reaction could be read from the point of the curve where amplifications of standard and target were equal.

The same method was used to determine the GAPDH mRNA levels in these total RNA preparations. The primer sequences are the following: 5′-GTCAGTGGTGGAC CTGA CCTG-3′ for the sense 1 primer, 5′-<u>GTCAGTGG TGGACCTGACCTG</u>ATTTGGCTACAGC AACAG GGTG-3′ for the sense 2 primer, and 5′-TGAGGAGG GGAGATTCAGTG-3′ for the antisense primer. The GAPDH standard was 190 bp in length versus 400 bp for the endogenous GAPDH sequences. Total RNA (125 ng) was reverse transcribed with the First Strand cDNA Synthesis Kit for RT-PCR and GAPDH antisense primer. Then, 2 μL (1/10) of the RT product was coamplified with 960, 480, 192, 96, and 48 fg of GAPDH standard and with the sense 1 and antisense GAPDH primers. The thermal cycling reactions were the same as for ICAM-1 mRNA analysis. The PCR products subjected to 1.5% agarose gel were analyzed as described below. The values obtained for GAPDH signals were used to normalize those for ICAM-1 signals.

Semiquantitative RT-PCR for VCAM-1 mRNA analysis. VCAM-1 mRNA levels were analyzed by semiquantitative RT-PCR. Both cDNA synthesis and PCR were performed in a single tube with the SuperScript™ One-Step RT-PCR kit according to the manufacturer's instructions. The primer sequences were the following: sense 5′-CCCTTGACCGGCTGGAGATTG-3′ and antisense 5′-CTGTGTCTCCTGTCTCC GCTT-3′ for VCAM-1 (the VCAM-1 cDNA obtained was 638 bp in length) and sense 5′-GAGTCAACGGATTTGGTCGT-3′ and antisense 5′-TTGATTTTGGAGGGATC-3′; for GAPDH (the GAPDH cDNA was 238 bp in length). The linearity of the amplification was determined for cycle number and RNA concentrations in preliminary experiments. For

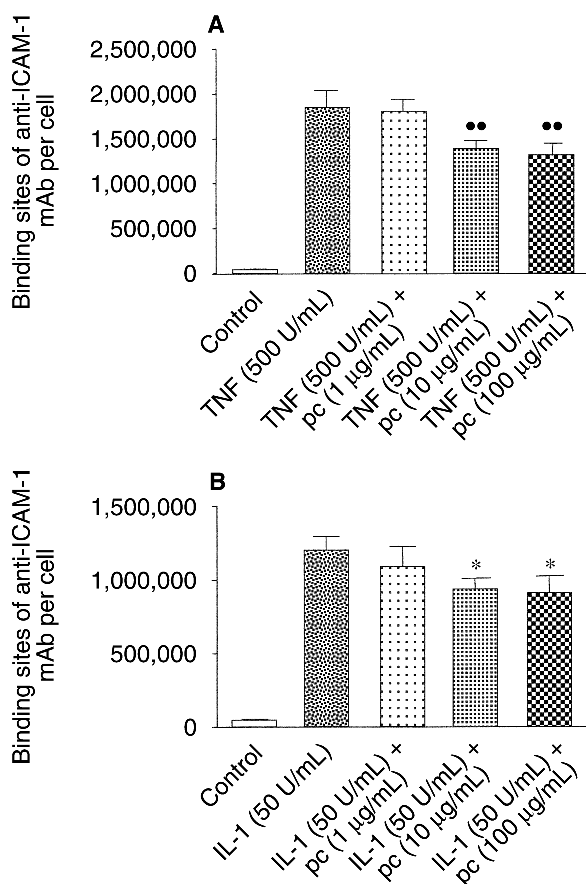

Fig. 1. Effects of p-cresol (pc) on intercellular adhesion molecule-1 (ICAM-1) expression on human umbilical vein endothelial cells (HUVEC) stimulated with tumor necrosis factor (TNF; *A*) or interleukin-1β (IL-1β; *B*). Endothelial cells were incubated for 24 hours in medium (control), TNF (500 U/mL), or IL-1β (50 U/mL) in the presence or absence of different concentrations of p-cresol. After incubation, endothelial expression of ICAM-1 was analyzed by flow cytometry after indirect immunofluorescence staining. Data are expressed as mean ± SEM of 6 independent experiments. ••$P < 0.01$ vs. TNF, *$P < 0.05$ vs. IL-1β.

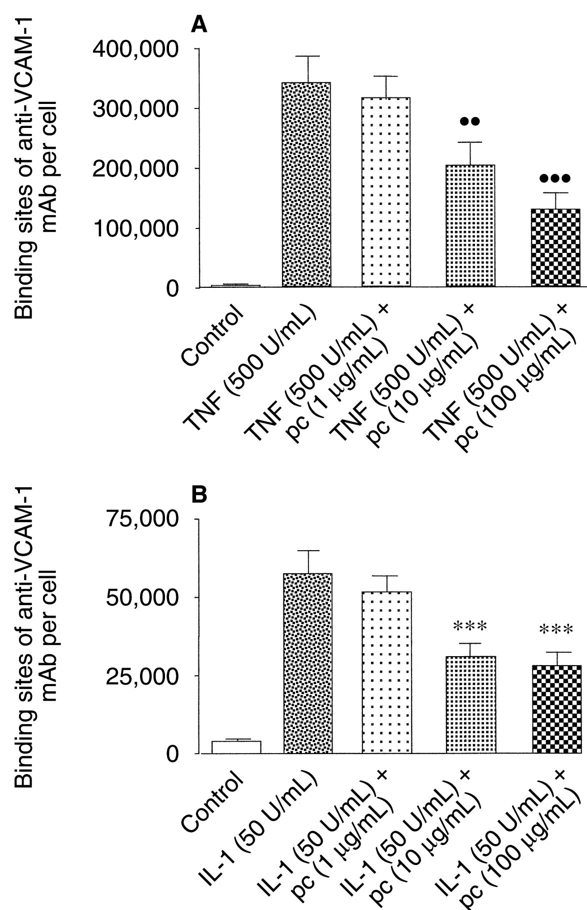

Fig. 2. Effects of p-cresol (pc) on VCAM-1 expression on HUVEC stimulated with TNF (*A*) or IL-1β (*B*). Endothelial cells were incubated for 24 hours in medium (control), TNF (500 U/mL), or IL-1β (50 U/mL) in the presence or absence of different concentrations of p-cresol. After incubation, endothelial expression of VCAM-1 was analyzed by flow cytometry after indirect immunofluorescence staining. Data are expressed as mean ± SEM of 6 independent experiments. ••$P < 0.01$ vs. TNF, •••$P < 0.001$ vs. TNF, ***$P < 0.001$ vs. IL-1β.

VCAM-1 mRNA analysis, 50 ng and 12.5 ng of total RNA were chosen for IL-1β and TNF stimulation, respectively. The thermal cycling reactions were denaturation at 94°C for 30 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 30 seconds (for a total of 20 cycles), followed by a termination step at 72°C for 10 minutes. For GAPDH mRNA, the amount of total RNA was 6 ng and the thermal cycling reactions were denaturation at 94°C for 30 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 30 seconds (for a total of 25 cycles). The RT-PCR products were subjected to 1.5% agarose gels. The intensity of the ethidium bromide staining for each band was densitometrically measured with the Scion Image software. All VCAM-1 signals were normalized with GAPDH signals and the results were expressed in arbitrary units.

Table 1. Effects of p-cresol on ICAM-1, VCAM-1 and E-selectin expression on cytokine-stimulated HUVEC after a 4-hour incubation

	ICAM-1	VCAM-1	E-selectin
Control	28 ± 8	5 ± 2	4 ± 2
TNF	418 ± 64	89 ± 7	150 ± 25
TNF + pc *10 μg/mL*	303 ± 25[a]	68 ± 6[b]	137 ± 27
TNF + pc *100 μg/mL*	287 ± 29[a]	56 ± 5[c]	139 ± 27
IL-1β	116 ± 18	58 ± 9	274 ± 12
IL-1β + pc *10 μg/mL*	79 ± 10[f]	39 ± 5[d]	269 ± 19
IL-1β + pc *100 μg/mL*	68 ± 14[f]	34 ± 7[e]	254 ± 16

Endothelial cells were incubated for 4 hours in medium (control), tumor necrosis factor (TNF 500 U/mL), or interleukin-1β (IL-1β 50 U/mL) in presence or absence of different concentrations of p-cresol (pc). After incubation, endothelial expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin was analyzed by flow cytometry after indirect immunofluorescence staining. Results are expressed as number of binding sites of mAb per cell divided by 10^3. Data are expressed as mean ± SEM of 7 independent experiments.

[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$ vs. TNF
[d] $P < 0.05$, [e] $P < 0.01$, [f] $P < 0.001$ vs. IL-1β

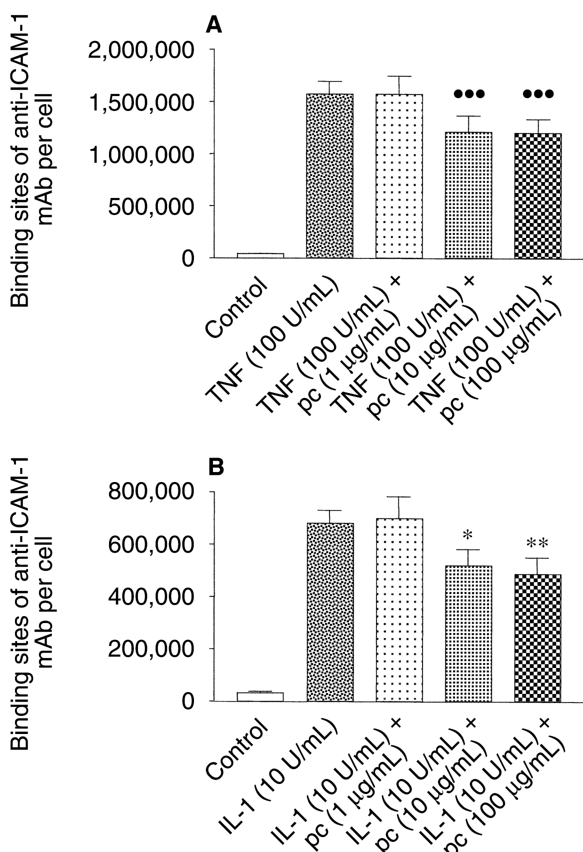

Fig. 3. Effects of p-cresol (pc) on ICAM-1 expression on HUVEC stimulated with lower doses of TNF (*A*) or IL-1β (*B*). Endothelial cells were incubated for 24 hours in medium (control), TNF (100 U/mL), or IL-1β (10 U/mL) in the presence or absence of different concentrations of p-cresol. After incubation, endothelial expression of ICAM-1 was analyzed by flow cytometry after indirect immunofluorescence staining. Data are expressed as mean ± SEM of 6 independent experiments. •••$P < 0.001$ vs. TNF, *$P < 0.05$ vs. IL-1β, **$P < 0.01$ vs. IL-1β.

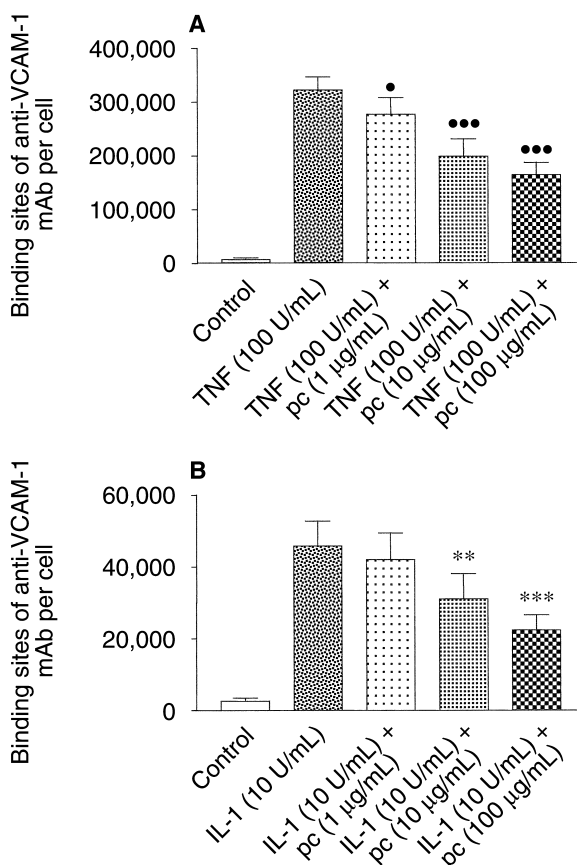

Fig. 4. Effects of p-cresol (pc) on VCAM-1 expression on HUVEC stimulated with lower doses of TNF (*A*) or IL-1β (*B*). Endothelial cells were incubated for 24 hours in medium (control), TNF (100 U/mL), or IL-1β (10 U/mL) in the presence or absence of different concentrations of p-cresol. After incubation, endothelial expression of VCAM-1 was analyzed by flow cytometry after indirect immunofluorescence staining. Data are expressed as mean ± SEM of 6 independent experiments. •$P < 0.05$ vs. TNF, •••$P < 0.001$ vs. TNF, **$P < 0.01$ vs. IL-1β, *** $P < 0.001$ vs. IL-1β.

Adhesion assay of THP-1 to HUVEC

Endothelial cells cultured in 96-well plates were incubated for 24 hours in EGM-2 medium containing different concentrations of p-cresol or containing methanol 1% (control), in the presence or absence of TNF (500 U/mL) or IL-1β (50 U/mL).

THP-1 cells were washed with RPMI medium and labeled with 10 μmol/L Calcein-AM at 37°C during 30 minutes. After three washes in RPMI, THP-1 cells were suspended in culture medium at 37°C at a concentration of 10^6/mL. Cell viability assessed by trypan blue exclusion was greater than 90%.

Adhesion of THP-1 to HUVEC was measured with the method of Vaporcyian, Jones and Ward [13]. One hundred microliters of THP-1 (10^6/mL) were added on washed HUVEC in triplicate and incubated for 30 minutes at 37°C in 5% CO_2 atmosphere. Culture medium alone was used as a blank. Total fluorescence (Ft) and fluorescence of blank (Fb_1) were measured. Non-adherent THP-1 cells were removed by three washings, and culture medium was added to each well. The remaining fluorescence (Fx) and the fluorescence of the blank (Fb_2) were measured, and percentage adhesion was calculated by the following formula:

$$\% \text{ adhesion} = [(Fx - Fb_2)/(Ft - Fb_1)] \times 100$$

Fluorescence was determined by the Cytofluor® Series 4000 Fluorescence multi-well plate reader (PerSeptive Biosystems, Framingham, MA, USA). The excitation filter was a 20 nm bandwidth centered at 485 nm and the emission filter was a 25 nm bandwidth centered at 530 nm. This method yielded low variation between replicates.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) of a minimum of three independent experi-

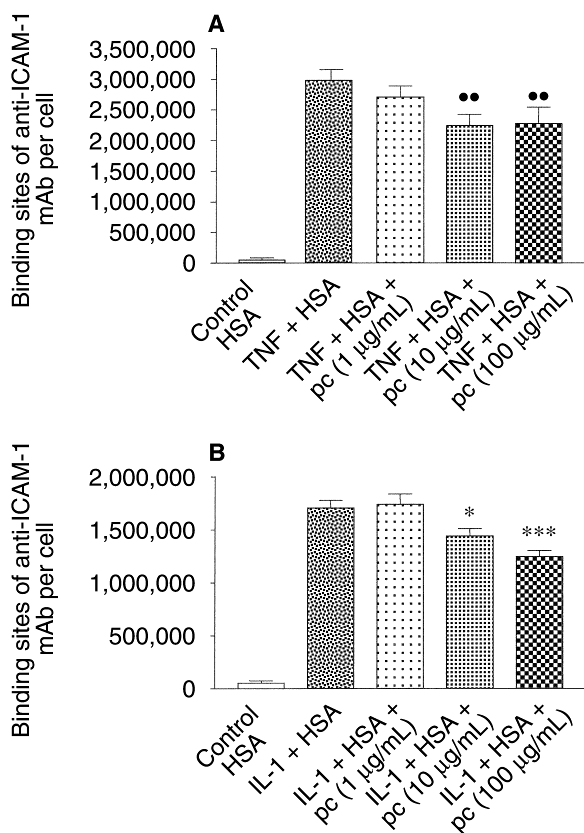

Fig. 5. Effects of p-cresol (pc) on ICAM-1 expression on HUVEC stimulated with TNF (*A*) or IL-1β (*B*) in presence of human serum albumin (HSA) at 4 g/dL. Endothelial cells were incubated for 24 hours in medium supplemented with 4 g/dL HSA (control), TNF (500 U/mL), or IL-1β (50 U/mL) in presence or absence of different concentrations of p-cresol. After incubation, endothelial expression of ICAM-1 was analyzed by flow cytometry after indirect immunofluorescence staining. Data are expressed as mean ± SEM of 5 independent experiments. ••$P < 0.01$ vs. TNF, *$P < 0.05$ vs. IL-1β, ***$P < 0.001$ vs. IL-1β.

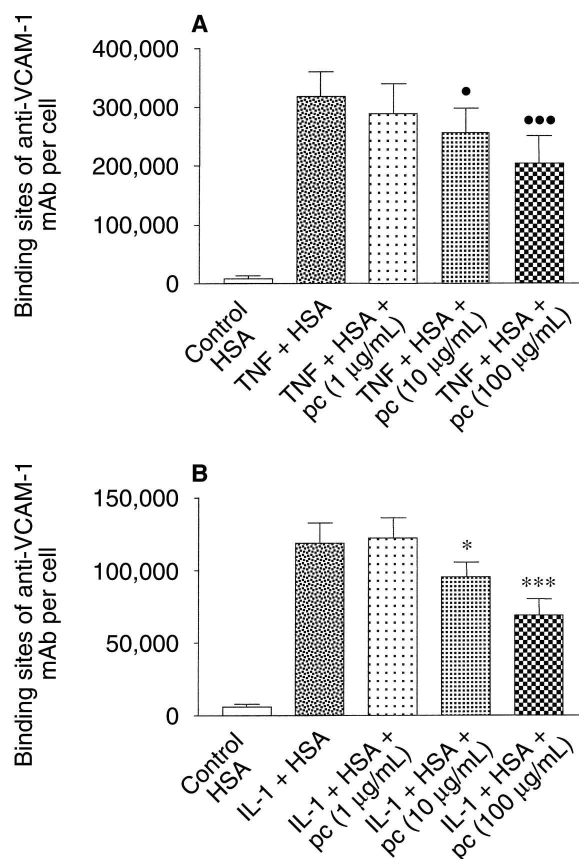

Fig. 6. Effects of p-cresol (pc) on VCAM-1 expression on HUVEC stimulated with TNF (*A*) or IL-1β (*B*) in presence of HSA at 4 g/dL. Endothelial cells were incubated for 24 hours in medium supplemented with 4 g/dL HSA (control), TNF (500 U/mL), or IL-1β (50 U/mL) in the presence or absence of different concentrations of p-cresol. After incubation, endothelial expression of VCAM-1 was analyzed by flow cytometry after indirect immunofluorescence staining. Data are expressed as mean ± SEM of 5 independent experiments. •$P < 0.05$ vs. TNF, •••$P < 0.001$ vs. TNF, *$P < 0.05$ vs. IL-1β, ***$P < 0.001$ vs. IL-1β.

ments. Statistical analysis was performed with the Prism® software (GraphPad Software Inc., San Diego, CA, USA). Determination of significant differences was performed by repeated measures analysis of variance (ANOVA) followed by a Tukey's test. A *P* value lower than 0.05 was considered significant.

RESULTS

P-cresol down-regulates cytokine-induced expression of endothelial adhesion molecules

Endothelial cells were incubated in medium with or without different concentrations of p-cresol, in presence or absence of TNF (500 U/mL) or IL-1β (50 U/mL). After 24 hours, ICAM-1 and VCAM-1 expression on endothelial cell surface was measured by flow cytometry. P-cresol alone had no effect on endothelial adhesion molecule expression whatever the dose used (not shown). After a 24-hour incubation, TNF and IL-1β induced a strong increase in ICAM-1 and VCAM-1 expression on endothelial surface (Figs. 1 and 2). The increase in ICAM-1 expression induced by TNF was inhibited by 27% by 10 μg/mL of p-cresol (Fig. 1). Similar results were obtained with IL-1β (Fig. 1). P-cresol at 100 μg/mL also inhibited ICAM-1 expression on cytokine-stimulated HUVEC but did not induce a stronger inhibition than p-cresol at 10 μg/mL (Fig. 1). P-cresol at 1 μg/mL (healthy subject concentration) did not significantly modify cytokine-induced ICAM-1 expression on HUVEC (Fig. 1). The increase in VCAM-1 expression was also inhibited by p-cresol; VCAM-1 induction by TNF and IL-1β was decreased 42% and 49%, respectively, by p-cresol at 10 μg/mL (Fig. 2). VCAM-1 expression on cytokine-stimulated HUVEC also was inhibited by p-cresol at 100 μg/mL; with TNF, this inhibition was stronger than with p-cresol at 10 μg/mL. VCAM-1 induction by TNF was decreased by 56% by p-cresol at 100 μg/mL (Fig. 2). P-cresol at

1 μg/mL had no effect on cytokine-induced VCAM-1 expression on HUVEC (Fig. 2).

Inhibition of cytokine-induced expression of ICAM-1 and VCAM-1 occurred rapidly, since the surface expression of these molecules also was inhibited by p-cresol after a four-hour incubation (Table 1).

Endothelial cells were stimulated with lower amounts of TNF and IL-1β (100 U/mL and 10 U/mL, respectively), and the inhibitory effect of p-cresol on cytokine-induced adhesion molecule expression was similar (Figs. 3 and 4). The increase in ICAM-1 expression induced by TNF at 100 U/mL was inhibited 22% and 23% by 10 μg/mL and 100 μg/mL of p-cresol, respectively (Fig. 3). VCAM-1 induction by TNF at 100 U/mL was decreased 38% and 49% by p-cresol at 10 μg/mL and 100 μg/mL, respectively (Fig. 4). Similar results were obtained with IL-1β at 10 U/mL (Figs. 3 and 4).

E-selectin was analyzed at its maximal level of surface expression, that is, after a four-hour incubation (Table 1). E-selectin expression on HUVEC incubated with TNF at 500 U/mL or IL-1β at 50 U/mL was strongly increased relative to control. Cytokine-induced E-selectin expression was not inhibited by p-cresol, whatever the dose (Table 1).

Since p-cresol is protein-bound, the influence of albumin concentration on adhesion molecule expression was analyzed. The albumin concentration in the usual culture medium was 0.08 g/dL. HSA was added to the culture medium at a concentration of 4 g/dL. At this albumin concentration, an inhibition of cytokine-induced expression of adhesion molecules was caused by p-cresol (Figs. 5 and 6). This inhibition was lower than that obtained with medium not supplemented with albumin: TNF-induced ICAM-1 expression was inhibited 22% and 23% by p-cresol at 10 μg/mL and 100 μg/mL, respectively (Fig. 5); TNF-induced VCAM-1 expression was inhibited 20% and 36% by p-cresol at 10 μg/mL and 100 μg/mL, respectively (Fig. 6). Similar results were obtained with IL-1β–induced adhesion molecule expression (Figs. 5 and 6). P-cresol at 1 μg/mL did not modify cytokine-induced adhesion molecule expression in these conditions (Figs. 5 and 6).

P-cresol inhibits cytokine-induced mRNA of endothelial adhesion molecules

To explore the mechanisms responsible for inhibition of ICAM-1 and VCAM-1 expression, we studied ICAM-1 and VCAM-1 mRNA levels by RT-PCR. These experiments were performed only with p-cresol concentrations inducing an inhibition of adhesion molecule expression, that is, 10 μg/mL and 100 μg/mL. P-cresol did not increase ICAM-1 (Fig. 7) and VCAM-1 (Fig. 8, lanes 1 and 2) mRNA levels in the absence of inflammatory stimuli. Stimulation of HUVEC with IL-1β or TNF resulted in a strong increase in ICAM-1 (Fig. 7) and VCAM-1 (Fig. 8A, lanes 3 and 6) mRNA. The increase in ICAM-1 and VCAM-1 mRNA induced by IL-1β was inhibited by p-cresol at 10 μg/mL and 100 μg/mL (Figs. 7A, 8A lanes 4 and 5, and 8B). Similar results were obtained when IL-1β was replaced by TNF (Figs. 7B, 8A lanes 7 and 8, and 8C).

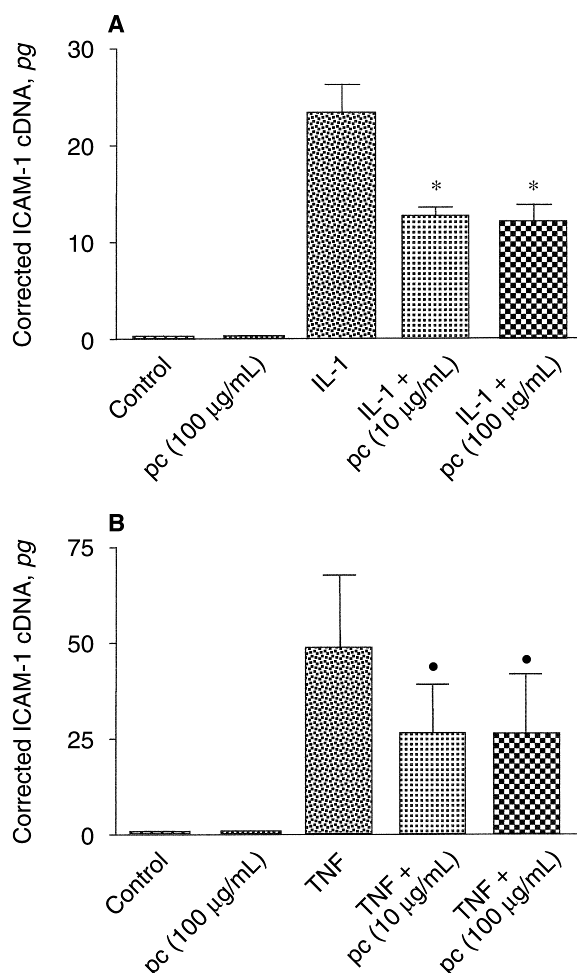

Fig. 7. Effects of p-cresol (pc) on ICAM-1 mRNA level in HUVEC stimulated with IL-1β (*A*) or TNF (*B*). Endothelial cells were incubated for 16 hours in control medium, IL-1β (50 U/mL), or TNF (500 U/mL) in the presence or absence of p-cresol. Total RNA was then isolated and ICAM-1 and GAPDH mRNA levels were analyzed by competitive RT-PCR as described in the **Methods** section. Intensities of the ethidium bromide staining of RT-PCR products subjected to 1.5% agarose gel were quantified with the Scion Image software. GAPDH signals were used to normalize ICAM-1 signals. Data are expressed as mean ± SEM of 3 independent experiments. *$P < 0.05$ vs. IL-1β, •$P < 0.05$ vs. TNF.

P-cresol reduces cytokine-induced THP-1 adhesion to endothelial cells

To determine the functional importance of the inhibitory properties of p-cresol on stimulated expression of endothelial adhesion molecules, we studied the adhesion of THP-1 monocytes to HUVEC. The stimulation of HUVEC by TNF or IL-1β resulted in a sixfold increase

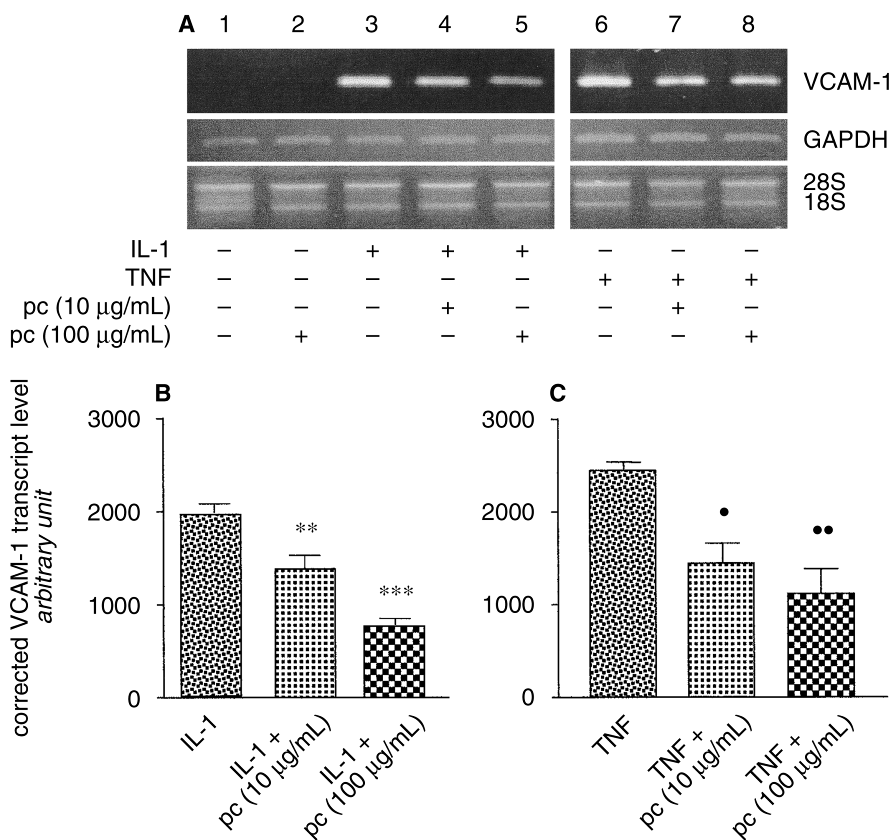

Fig. 8. Effects of p-cresol (pc) on VCAM-1 mRNA level in HUVEC stimulated with IL-1β or TNF. Endothelial cells were incubated for 16 hours in control medium, IL-1β (50 U/mL), or TNF (500 U/mL), in the presence or absence of p-cresol. After incubation, VCAM-1 and GAPDH mRNA levels in HUVEC were analyzed by semiquantitative RT-PCR. (*A*) UV-illuminated gel electrophoresis from a representative experiment. (*B* and *C*) The mean ± SEM of band intensities of VCAM-1 cDNA from 5 independent experiments, after normalization with the intensities of GAPDH cDNA. The results are expressed in arbitrary units. **$P < 0.01$ vs. IL-1β, ***$P < 0.001$ vs. IL-1β, •$P < 0.05$ vs. TNF, ••$P < 0.01$ vs. TNF.

in THP-1 adhesion (Fig. 9); treatment with p-cresol alone did not modify THP-1 adhesion, whatever the dose of p-cresol used (not shown). Incubation with p-cresol significantly reduced THP-1 adhesion to cytokine-stimulated HUVEC. P-cresol at 10 μg/mL decreased monocyte adhesion to TNF and IL-1β–stimulated HUVEC by 25% and 42%, respectively (Fig. 9). P-cresol at 100 μg/mL also strongly decreased monocyte adhesion to stimulated HUVEC (Fig. 9). P-cresol at 1 μg/mL had no effect on monocyte adhesion to TNF-stimulated HUVEC, but slightly decreased monocyte adhesion to IL-1β–stimulated HUVEC (Fig. 9).

DISCUSSION

Upon appropriate stimulation, the endothelium exhibits increased adhesiveness for leukocytes. Increased expression of endothelial-leukocyte adhesion molecules (such as ICAM-1, VCAM-1 and E-selectin) mediates such enhanced adhesive interactions [14, 15]. Inflammatory cytokines and leukocyte-specific chemoattractants also contribute to leukocyte recruitment during inflammatory and immune responses [16, 17]. Since p-cresol plasma levels are enhanced in CRF patients and since p-cresol may play a role in the immunodeficiency of uremia, we hypothesized that p-cresol could modulate the expression of endothelial adhesion molecules. We investigated the effects of p-cresol on endothelial adhesion molecules during cytokine stimulation, since most of the endothelial molecules mediating leukocyte adhesion are strongly inducible.

The results demonstrated that p-cresol significantly inhibited cytokine-stimulated expression of endothelial adhesion molecules ICAM-1 and VCAM-1. P-cresol alone had no effect on endothelial adhesion molecule expression. Cell adhesion molecules were inhibited at a p-cresol concentration similar to that in uremic plasma [8, 18]. On the contrary, p-cresol at concentrations observed in the plasma of healthy subjects had no notable effect on induction of endothelial adhesion molecules. At the transcriptional level, the mRNA levels of VCAM-1 and ICAM-1 decreased when cytokine-stimulated endothelial cells were exposed to p-cresol.

Since p-cresol is protein-bound in plasma, the culture medium was supplemented with albumin at a concentration found in human plasma. In the presence of albumin, p-cresol inhibited cytokine-induced adhesion molecule expression, but to a lesser extent than in medium not supplemented with albumin. This could be explained by the decrease of the free non-protein bound fraction of p-cresol in medium. It is difficult to extrapolate these in vitro results to the in vivo situation. However, they show

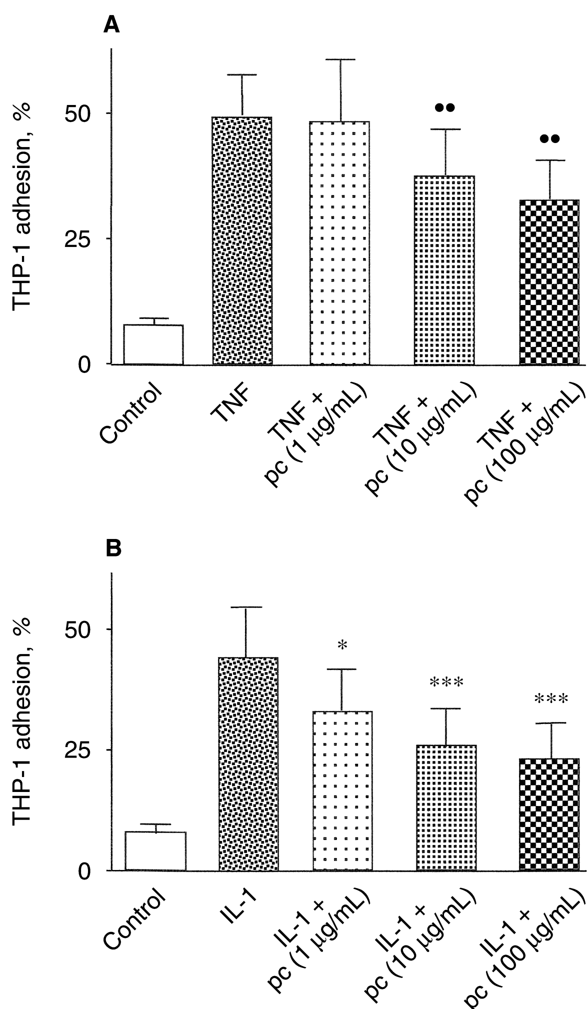

Fig. 9. Effects of p-cresol (pc) on THP-1 adhesion on HUVEC stimulated with TNF (*A*) or IL-1β (*B*). Endothelial cells were incubated for 24 hours in medium (control), TNF (500 U/mL), or IL-1β (50 U/mL) supplemented or not with different concentrations of p-cresol. After incubation, THP-1 cells labeled with calcein-AM were added, and THP-1 adhesion to endothelial cells was analyzed. Data are expressed as mean ± SEM of 10 independent experiments for TNF and 7 independent experiments for IL-1β. ••$P < 0.01$ vs. TNF, *$P < 0.05$ vs. IL-1β, ***$P < 0.001$ vs. IL-1β.

that at a uremic concentration, and in presence of albumin at concentration found in human plasma, p-cresol has an effect on endothelial cells.

The three endothelial adhesion molecules have different patterns of expression and are functionally distinct. E-selectin mediates the initial leukocyte rolling on endothelium whereas ICAM-1 and VCAM-1 mediate firm adhesion by interacting with integrins on the leukocyte surface. The expression of endothelial adhesion molecules is strongly up-regulated by TNF and IL-1β at the transcriptional level through the activation of the NF-κB system. Although the endothelial NF-κB transcription factor system is necessary for cytokine-induced endothelial adhesion molecule expression, it is not sufficient. Other transcription factors must assemble with NF-κB to generate unique transcriptional activating complexes. For example, the high mobility group (HGM) protein I(Y) is required for cytokine-induced E-selectin expression, and the transcriptional activator interferon regulatory factor-1 (IRF-1) cooperates with NF-κB to mediate cytokine induction of VCAM-1 [19]. On cytokine stimulated endothelial cells, p-cresol inhibited ICAM-1 and VCAM-1 up regulation, but not E-selectin induction, suggesting that p-cresol acts preferentially on ICAM-1 and VCAM-1. It remains unclear how p-cresol inhibits cytokine-stimulated VCAM-1 and ICAM-1 expression without inhibiting E-selectin expression. One possible mechanism is that p-cresol acts on transcriptional factors involved in VCAM-1 and ICAM-1 induction but not in E-selectin induction. In addition, stimulated levels of VCAM-1 transcript are reduced in the presence of the protein synthesis inhibitor cycloheximide [19], indicating that protein synthesis is necessary for maximal transcriptional activation. In contrast, E-selectin is superinduced in the presence of the protein synthesis inhibitor [19]. Hence, another possible mechanism is that p-cresol inhibits the synthesis of proteins necessary for VCAM-1 mRNA induction.

The molecular mechanisms of p-cresol action on endothelial cells are not known. A marked impairment of ICAM-1 mRNA expression was shown in TNF-stimulated endothelial cells depleted of intracellular glutathione [20]. In addition, previous experiments on rat liver cells demonstrated that p-cresol depletes intracellular glutathione levels and causes toxicity via the formation of a reactive intermediate that can be trapped with glutathione [21]. Therefore, we speculate that p-cresol may act on endothelial cells by modifying intracellular endothelial glutathione levels. Further studies are necessary to confirm this hypothesis.

Since leukocyte adhesion to endothelium is one of the key events in immune responses, we studied monocyte adhesion to endothelial cells. P-cresol alone had no effect on monocyte adhesion to non-stimulated endothelial cells. On the other hand, uremic levels of p-cresol significantly reduced monocyte adhesion to cytokine-stimulated endothelial cells. Hence, the decrease by p-cresol of adhesion molecule expression on stimulated endothelial cells is of functional importance because it results in an inhibition of monocyte adhesion to these endothelial cells.

The functional state of leukocytes obtained from undialyzed uremic patients or during a dialysis session is impaired [22–27]. P-cresol may be involved in uremic leukocyte functional deficiency since it depresses the phagocyte functional capacity [5] and inhibits the release of PAF by macrophages [6]. In the present study, we demonstrated that p-cresol also has a harmful effect on leukocyte/endothelial cell interactions.

Only 30% of p-cresol is removed by hemodialysis ther-

apies, which eliminate over 70% of urea and creatinine [28], probably because most p-cresol is protein-bound. No difference in p-cresol removal was found between synthetic and cellulosic membranes, or low flux and high flux membranes [28]. Whereas dialyzers containing membranes with a larger pore size remove middle molecules better, a larger pore size has no effect on the removal of protein-bound uremic toxins such as p-cresol. Therefore, other solutions should be developed to remove p-cresol, such as dialysis membranes with higher adsorption. An alternative solution is to reduce the intestinal load of p-cresol, for example with the uptake of the oral sorbent AST-120 [29, 30].

Endothelial alteration in uremia has previously been demonstrated in vivo and in vitro. In CRF patients, endothelium-dependent vasodilation is altered [31], and plasma levels of soluble endothelial molecules [32–35] are increased. In vitro, uremic medium modifies endothelial functions [36–38], and uremic toxins induce endothelial alteration. Homocysteine alters the expression of multiple genes in endothelial cells [39], oxalate inhibits endothelial cell replication and migration [40], and AGEs increase endothelial permeability [41] and up-regulate the expression of endothelial adhesion molecules [42]. The present study revealed that p-cresol is a new uremic toxin that plays a role in endothelial alterations in uremia. The in vitro effect of p-cresol is just the opposite of the effect of AGEs. This suggests that the various compounds circulating in the plasma of uremic patients may either amplify the recruitment of inflammatory cells or prevent the recruitment of immunologic competent cells.

In conclusion, we demonstrate that p-cresol inhibits both cytokine-induced expression of endothelial adhesion molecules and stimulated monocyte adhesion to endothelial cells. Since leukocyte adhesion to vascular endothelial cells is a critical process in defense mechanisms, the effects of p-cresol could be involved in the immune defect of CRF patients.

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