Clindamycin Modulates Inflammatory-Cytokine Induction in Lipopolysaccharide-Stimulated Mouse Peritoneal Macrophages

Tetsuji Nakano, Kazufumi Hiramatsu, Kenji Kishi, Norio Hirata, Jun-ichi Kadota, and Masaru Nasu*

Second Department of Internal Medicine, Oita Medical University, Hasama, Oita 879-5593, Japan

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We investigated the mechanism by which clindamycin (CLI) modulates cytokine induction after lipopolysaccharide (LPS) stimulation. Although CLI decreased the intracellular expression levels of tumor necrosis factor alpha and interleukin 1β (IL-1β) concentrations and increased IL-6 expression in macrophages, cytokine mRNA expression levels were similar in CLI-treated and untreated groups. Our findings suggest that CLI modulates cytokine production in LPS-stimulated macrophages.

Anticytokine effects, which are independent of their antibacterial properties, have been reported elsewhere for several antimicrobial agents (2, 7, 9). Furthermore, these anticytokine effects on inflammatory cytokines have been studied at a molecular level (12, 14, 16). It was recently reported that clindamycin (CLI) reduces tumor necrosis factor alpha (TNF-α) concentrations in lipopolysaccharide (LPS)-stimulated THP-1 cells (8) and that CLI decreases TNF-α and interleukin 1β (IL-1β) concentrations and increases serum IL-6 concentrations, as well as reducing mortality in the mouse model (4). The present study showed the mechanism of modulation by CLI of inflammatory-cytokine production by LPS-stimulated macrophages, both in vitro and in vivo.

Two milliliters of 4% thioglycolate fluid medium (Difco, Detroit, Mich.) was intraperitoneally injected into 10-week-old C3H/HeN male mice. After 4 days peritoneal lavage fluid was collected and cultured on plastic plates in Iscove’s modified Dulbecco’s medium supplemented with 10% heat-inactivated fetal bovine serum at 37°C under 5% CO2 for 90 min. Adherent cells were used as the macrophages (esterase staining confirmed that 95% ± 2% of the cells were macrophages). The cells (5 x 105 cells/well) in the medium were pretreated for 30 min with CLI (5 to 100 µg/ml), which is available as Dalacin S injectable (Pharmacia & Upjohn, Tokyo, Japan), or not, cells were stimulated with purified Escherichia coli O55:B5 LPS (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 100 ng/ml for 2 or 4 h. The culture supernatants were collected and used for enzyme-linked immunosorbent assay (ELISA), and adherent cells were used for RNA extraction and flow cytometry analysis.

In the in vitro study, 30 min after the administration of CLI at 160, 300, or 440 mg/kg of body weight or of saline as a control, 40 µg of purified LPS/kg was injected intraperitoneally into the mice. After 2 or 6 h, peritoneal lavage fluid was collected and cultured with the medium for 45 min on plastic plates. Adherent cells were used as the macrophages for RNA extraction and flow cytometry analysis.

TNF-α, IL-1β, and IL-6 concentrations in the culture supernatant were measured with an ELISA kit (Cytoscreen; BioSource International, Camarillo, Calif.). TNF-α concentrations in the supernatants of cells pretreated with 25 and 100 µg of CLI/ml were 718 ± 83 and 675 ± 76 pg/ml, respectively, while the concentration was 1,080 ± 107 pg/ml in the control group (P < 0.05) after 2 h of stimulation with LPS (Fig. 1A). In the same groups, IL-1β concentrations were significantly reduced to 289 ± 13 and 285 ± 25 pg/ml, respectively, compared with the control group (344 ± 19 pg/ml; P < 0.05), after 4 h of LPS stimulation (Fig. 1B). On the other hand, the concentrations of IL-6 in the CLI-pretreated groups increased in a dose-dependent manner (Fig. 1C). No increase in cytokine concentration was seen for supernatants of macrophages treated with CLI alone (data not shown).

Reverse transcription-PCR was performed in order to compare the expression levels of TNF-α, IL-1β, and IL-6 mRNAs. Total RNA was extracted from 5 x 106 macrophage cells with Isogen (Nippon Gene Co., Tokyo, Japan), in both in vitro and in vivo experiments. cDNA was synthesized from 1 µg of total RNA with oligo(dT)12–18 primers (Gibco-BRL, Gaithersburg, Md.), Moloney murine leukemia virus reverse transcriptase (Gibco-BRL), RNase inhibitor (Toyobo Biochemicals, Osaka, Japan), and deoxynucleoside triphosphates. PCR was performed with 5 µl of cDNA, each primer, and EX Taq DNA polymerase (Takara Shuzo, Kyoto, Japan). The PCR profile was 25 cycles of 1 min at 94°C, 2 min at 55°C, and 1 min at 72°C followed by 10 min at 72°C. The primers for β-actin, IL-1β, and IL-6 were used as described previously (10), and those for TNF-α were as follows: sense, 5‘-CGAGTGACAAGCCTGTAGCC-3’, and antisense, 5‘-GCAATTGCATCTAAGTAGA-3’ (3). In the in vitro study, mRNA expression levels in macrophages of the above cytokines after 2 or 4 h of LPS stimulation were higher than those in the non-LPS-stimulated groups. Pretreatment of LPS-stimulated macrophages with CLI (5 to 100 µg/ml) had no effect on cytokine mRNA expression (Fig. 2). In addition, no bands were detected in LPS-
stimulated and unstimulated groups when PCR was performed for 23 cycles, but strong bands appeared when the PCR was performed for more than 30 cycles (data not shown). We also conducted in vivo experiments, since cytokine concentrations may be affected by other factors in the mouse model. The expression levels of cytokine mRNAs in peritoneal macrophages in mice stimulated with LPS for 2 or 6 h were compared, because the peak concentrations of TNF-α and IL-6 in serum had been previously observed at 2 h while that of IL-1β was observed at 6 h after the administration of LPS (4). Pretreatment with CLI had no effect on mRNA cytokine expression at any concentration in LPS-stimulated mice (Fig. 3). These results suggest that CLI alters inflammatory-cytokine production by LPS-stimulated macrophages after gene transcription.

We examined intracellular expression of the cytokines after LPS stimulation by flow cytometry. Adherent macrophage cells ($5 \times 10^5$) were fixed and permeabilized with Intraprep (Beckman Coulter, Tokyo, Japan). For staining of intracellular cytokines, phycoerythrin-conjugated anti-mouse TNF-α and IL-6 antibodies (BD PharMingen, San Diego, Calif.) were used, with phycoerythrin-conjugated anti-rat immunoglobulin G1

![Graphs showing kinetics of cytokine concentrations in culture supernatants of mouse peritoneal macrophages.](http://aac.asm.org/)

FIG. 1. Kinetics of cytokine concentrations in culture supernatants of mouse peritoneal macrophages. Mouse peritoneal macrophages were pretreated with CLI for 0.5 h, and then the culture supernatant was collected from the control group (open squares) and the groups pretreated with 5, 25, or 100 µg of CLI per ml (open circles, closed squares, or closed circles, respectively) at 2 or 4 h after stimulation with 100 ng of purified E. coli LPS/ml. Concentrations of TNF-α (A), IL-1β (B), and IL-6 (C) in the culture supernatant were measured by ELISA in two wells. Data are means ± standard deviations of four independent experiments. *, P < 0.05 relative to the control group by one-way analysis of variance and the Bonferroni-Dunn test.

![Graph showing effects of CLI on the mRNA expression of inflammatory cytokines in mouse peritoneal macrophages.](http://aac.asm.org/)

FIG. 2. Effects of CLI on the mRNA expression of inflammatory cytokines in mouse peritoneal macrophages in vitro. After pretreatment with CLI at the concentrations indicated in the figure for 0.5 h, the cells were stimulated with 100 ng of E. coli LPS per ml (+) for 2 or 4 h or not stimulated (−). Data are from an experiment representative of four independent experiments.
antibody (BD PharMingen) as the isotype control. Anti-mouse
IL-1β antibody (R&D Systems, Minneapolis, Minn.) labeled
with fluorescein isothiocyanate (FITC) by use of the Fluoro-
porter FITC protein labeling kit (Molecular Probes, Eugene,
Oreg.) was used, with FITC-conjugated anti-rat immunoglob-
ulin G1 antibody (R&D Systems) being used as the isotype
control for staining. Measurements were performed on 10⁶
cells with a FACS Calibur version 1.0 cell sorter (Becton Dick-
inson, San Jose, Calif.). In the in vitro study, CLI (5 to 100
μg/ml) reduced the proportions of cells positive for intracellu-
lar TNF-α and IL-1β in a dose-dependent fashion (Fig. 4A and
B). In contrast, the proportions of IL-6-positive cells were
higher in macrophages pretreated with 25 and 100 μg of CLI
per ml than in the control (Fig. 4C). In CLI-pretreated mice,
the proportions of cells positive for TNF-α (Fig. 5A) and IL-1β
(Fig. 5B) tended to decrease with increased CLI dose in a
dose-dependent manner. In contrast, in CLI-pretreated mice,
the proportions of cells positive for IL-6 (Fig. 5C) in macro-
phages tended to be higher as the CLI dose increased in
comparison with the control group.

Our in vitro studies revealed that CLI dose-dependently
decreased TNF-α and IL-1β production and increased IL-6

FIG. 3. Effects of CLI on TNF-α, IL-1β, and IL-6 mRNA expression in peritoneal macrophages in the LPS-stimulated mouse model. After
pretreatment with CLI at the dose indicated in the figure for 0.5 h, mice received 40 μg of purified E. coli LPS/kg (+) or no LPS (−). Peritoneal
macrophages were collected 2 or 6 h after administration of LPS. The cells from mice stimulated for 2 h were used for TNF-α and IL-6 assays (A),
while cells from mice stimulated for 6 h were used for the IL-1β assay (B). The same types of results were obtained from each of four independent
experiments.

FIG. 4. Effects of CLI on intracellular cytokine expression in mouse peritoneal macrophages in vitro. After pretreatment with CLI at the
concentrations indicated in the figure for 0.5 h, the cells were stimulated with 100 ng of E. coli LPS/ml (+) for 2 or 4 h or not stimulated (−).
The cells stimulated for 2 h were used for TNF-α and IL-6 assays, while cells stimulated for 4 h were used for the IL-1β assay. The bar graphs show
the means ± standard deviations of the proportions of cells positive for TNF-α, IL-1β, and IL-6 in the four independent experiments. *, P < 0.05
relative to the control group by one-way analysis of variance and the Bonferroni-Dunn test.
production by macrophages. These results are similar to those obtained in in vitro studies where LPS-stimulated human monocytes were treated with fosfomycin (11) and erythromycin (1, 5). In addition to the in vitro results, we confirmed that, in vivo, CLI modulated the induction of inflammatory cytokines through its action on peritoneal macrophages as one of the target cells.

Previous studies have demonstrated that LPS is involved in the induction of TNF-α at the transcriptional level (15) and that LPS antagonists inhibit TNF-α and IL-1β mRNA expression of LPS-stimulated monocytes (13). Tetracycline inhibited TNF-α and IL-1β production at a posttranscriptional level by LPS-stimulated human monocytes (16). Treatment with tetracycline inhibited TNF-α, IL-1β, and IL-6 mRNA expression levels in LPS-stimulated human peripheral blood cells (14). In the present study, CLI dose-dependently reduced TNF-α and IL-1β concentrations and dose-dependently increased IL-6 concentration in culture supernatants. On the other hand, mRNA expression levels of TNF-α, IL-1β, and IL-6 were similar regardless of CLI treatment of macrophages stimulated with LPS both in vitro and in the LPS-stimulated mice. These results suggest the involvement of CLI at translational or protein secretion levels. However, macrophage intracellular cytokine concentrations of TNF-α and IL-1β decreased, whereas that of IL-6 increased with increased dose of CLI both in vitro and in LPS-stimulated mice, suggesting that the effect of CLI may occur at the translational level for TNF-α, IL-1β, and IL-6 both in vitro and in vivo. Although clinical application is not feasible because of the high dosage of CLI used in vivo experiments, 25-μg/ml concentrations of CLI, which are clinically attainable in lung tissue (6), significantly modulated the concentrations of the cytokines in vitro experiments.

These findings suggest that CLI may be useful for the prevention of endotoxic shock.

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REFERENCES