The Effect of Mesoporphyrin on the Production of Cytokines by Inflammatory Cells In Vitro

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ABSTRACT—This study was conducted to investigate a mechanism of the anti-inflammatory action of mesoporphyrin, especially the effect on the production of cytokines by some cultured inflammatory cells. Mesoporphyrin had no effect on lipopolysaccharide-induced tumor necrosis factor- α production by RAW 264.7 cells (murine macrophage-like cells). Mesoporphyrin inhibited interferon- γ production by 1E10.H2 cells (murine T helper-1 cells), but not interleukin-4 production by D10.G4.1 cells (murine T helper-2 cells). Mesoporphyrin inhibited interleukin-6 production by human osteoblast-like MG-63 cells. This inhibition of interleukin-6 production is closely related to the suppression of prostaglandin E_2 generation by interfering cyclooxygenase 1 and 2 enzyme activities. These data suggest that the inhibition of cytokine production is one of the anti-inflammatory mechanisms of mesoporphyrin.

Keywords: Mesoporphyrin, Interferon- γ , Cyclooxygenase activity

Some porphyrin compounds are known to have anti-inflammatory and anti-allergic activities in vivo (1–3), and they have been used for the treatment of inflammatory diseases such as hepatitis and gastritis (4, 5) in humans. Mesoporphyrin is one of the porphyrin derivatives, and we have previously reported an anti-inflammatory action of mesoporphyrin on collagen-induced arthritis and superantigen-potentiated collagen-induced arthritis in mice (6).

Rheumatoid arthritis (RA) is an autoimmune disease characterized by severe inflammation of the joints. Many kinds of cells and mediators including cytokines participate in the onset of joint inflammation. In the synovial membrane of RA patients, several populations of circulating mononuclear cells migrate to the synovial lining, resulting in the formation of hyperplasia (7, 8). A number of cells including T cells, B cells, macrophages and fibroblasts are dispersed throughout the synovium (9–11) and accumulate within the lining layer (12, 13). The activation of synovium cells including osteoblasts leads to a severe inflammatory response in the joint.

In correspondence to cellular activation, the elevation of the level of cytokines such as interleukin-1 (IL-1)- β , tumor necrosis factor (TNF)- α and IL-6 in synovial fluids of RA patients have been reported (14–19). Moreover, some investigators have reported an abnormality of T cells, especially helper T cells, in the onset of RA (9–13,

20, 21). From the observations, helper T cells have classified into two subsets, Th1 and Th2 cells (22, 23). Th1 and Th2 cells were characterized by the production of interferon γ (IFN- γ) and IL-4, respectively. These evidences indicate an important role of the above-mentioned cytokines in the onset of RA. The present study was, therefore, conducted to investigate the effects of mesoporphyrin on the production of cytokines, including TNF- α , IFN- γ , IL-4 and IL-6, by some kinds of inflammatory cells.

MATERIALS AND METHODS

Drugs

Mesoporphyrin and indomethacin were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, USA), and prednisolone phosphate was purchased from Banyu Pharmaceutical Co., Ltd. (Tokyo), respectively. *N*-[2-Cyclohexyloxy-4-nitrophenyl] methanesulfonamide (NS-398) was kindly provided by Taisho Pharmaceutical Co., Ltd. (Saitama). Mesoporphyrin, indomethacin and NS-398 were dissolved in dimethyl sulfoxide (DMSO) before used.

TNF- α production by RAW264.7 cells

RAW264.7 cell, a murine macrophage-like cell, was obtained from American Type Culture Collection

(ATCC, Rockville, MD, USA). Cells were maintained in MEM (Gibco BRL, Berlin, Germany) containing 10% heat-inactivated fetal calf serum (FCS; Nippon Bio-Supp. Center, Tokyo), 55 mM 2-mercaptoethanol (2-ME, Sigma Chemical Co., Ltd.), 2 mM glutamine (Kishida Chemicals, Osaka), 10 mM non-essential amino acid (Gibco BRL), 100 U/ml penicillin (Banyu Pharmaceutical Co.) and 100 µg/ml streptomycin (Gibco BRL). RAW 264.7 cells (4×10^5 cells/ml) in 250 µl of culture medium were seeded into 48-well plates (Sumilon 48-well; Sumitomo Bakelite Co., Ltd., Osaka). After 24 hr, RAW264.7 cells were stimulated with 2.0 µg/ml lipopolysaccharide (LPS; Difco, Detroit, MI, USA) in 125 µl of culture medium and a 125-µl aliquot of drug solution was added. After cells were cultured for 48 hr at 37°C in a humidified atmosphere of 95% air 5% CO₂, the supernatant was removed and stored at -80°C until use.

IFN-γ production by 1E10.H2 cell

1E10.H2 cell, a murine Th1 cell line, was donated by Dr. H. Omori at Okayama University and maintained in medium consisting of 40% RPMI (Gibco BRL) and 40% DMEM (Gibco BRL) supplemented with 10% FCS, 10% cell-free supernatant from Con A (Sigma Chemical Co., Ltd.)-stimulated rat splenocyte (RCS), 2 mM glutamine, 1 mM sodium pyruvate (Gibco BRL), 55 mM 2-ME, 100 U/ml penicillin and 100 µg/ml streptomycin. 1E10.H2 cells were stimulated once a week with 50 µg/ml of key-hole limpets hemocyanin (Sigma Chemical Co., Ltd.) in the presence of mitomycin C (Wako Chemicals, Tokyo)-treated splenocytes from C3H/HeN mice (Japan SLC, Shizuoka) as antigen-presenting cells (APC). 1E10.H2 cells (3×10^5 cells/ml) in 200 µl RPMI supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin, were seeded into 48-well plates. 1E10.H2 cells were stimulated with 7.5 µg/ml Con A in 100 µl of the medium and 125 µl of drug solution was added. After cells were cultured for 48 hr at 37°C in a humidified atmosphere of 95% air 5% CO₂, the supernatants were centrifuged and stored at -80°C until the amount of IFN-γ was measured.

IL-4 production by D10.G4.1 cell

D10.G4.1 cell, a murine Th2 cell line, was donated by Taiho Pharmaceutical Co., Ltd. (Saitama). D10.G4.1 cells were maintained in the medium containing Click's medium (Irvine Scientific, Sant Ana, CA, USA) supplemented with 10% FCS, 10% RCS, 20 mg/ml α-methyl-D-mannoside (Nacalai Tesque, Kyoto), 2 mM glutamine, 50 mM 2-ME, 100 U/ml penicillin and 100 µg/ml streptomycin. D10.G4.1 cells were stimulated once a week with 100 µg/ml of conalbumin (Sigma Chemical Co., Ltd.) in the presence of mitomycin C-treated splenocytes

from AKR/J mice (Japan SLC) as APC. D10.G4.1 cells (3×10^5 cells/ml) in 200 µl of Click's medium supplemented with 10% FCS, 2 mM glutamine, 50 mM 2-ME, 100 U/ml penicillin and 100 µg/ml streptomycin, were seeded into 48-well plates. D10.G4.1 cells were stimulated by 10 µg/ml Con A in 100 µl of medium and 125 µl of drug solution was added. After cells were cultured for 48 hr at 37°C in a humidified atmosphere of 95% air 5% CO₂, the supernatants were centrifuged and stored at -80°C until the amount of IL-4 was measured.

IL-6 and prostaglandin E₂ (PGE₂) production by MG-63 cells

MG-63 cells, a human osteoblast-like line, was obtained from ATCC. MG-63 cells were maintained in MEM containing 10% FCS, 4 mM glutamine, 10 mM non essential amino acid, 100 U/ml penicillin and 100 µg/ml streptomycin. MG-63 cells (8×10^4 cells/ml) in 250 µl of culture medium were seeded into 48-well plates for 24 hr at 37°C in a humidified atmosphere of 95% air 5% CO₂. Then, MG-63 cells were stimulated with 2.0 ng/ml IL-1β (Genzyme, Cambridge, MA, USA) in 125 µl of culture medium and a 125 µl drug solution was added. From the results of preliminary experiments, the production of PGE₂ reached the peak at 48 hr, and IL-6 reached the peak at 96 hr after the stimulation of IL-1β. Supernatants were obtained at 48 hr for PGE₂ and at 96 hr for IL-6 and stored at -80°C until the measurement of the amount of PGE₂ and IL-6, respectively.

Measurement of the amount of cytokines and PGE₂

The amount of TNF-α, IFN-γ, IL-4 and IL-6 in the supernatant was assayed by using commercially available Enzyme Linked Immunosorbent assay (ELISA) kits (Endogen Inc., Woburn, MA, USA) and Enzyme Immunoassay (EIA) kit (Cayman Chemical Co., Ann Arbor, MI, USA) for PGE₂.

RNA isolation and RT-PCR analysis

RNA was extracted using ISOGEN (Nippon Gene Co., Ltd., Toyama), and all the procedures for the extraction of RNA were performed according to the manufacturer's instructions. RNA precipitates were washed with 1 ml of 75% ethanol, suspended in 0.5 ml of RNase-free H₂O and frozen at -80°C until use. The amount of total RNA in each sample was measured spectrophotometrically at 260 nm (Gene Quant, Cambridge, UK), and RNA quality was checked by electrophoresis. First strand cDNA was prepared using 1 µg RNA, superscript II reverse transcriptase and random hexamers (both Gibco BRL) according to the manufacturer's instructions. In brief, 12-µl reactions containing 1 µg RNA and random hexamers was heated for 10 min at 70°C and immediately chilled on ice.

The reaction was first preincubated for 5 min at 42°C with a mixture containing 2 μ l of 10 $\times$ buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 2 μ l 25 mM MgCl₂, 1 μ l dNTP mix (10 mM) and 2 μ l of 0.1 M DTT. Superscript reverse transcriptase (200 U) was added, and the mixture was incubated at 42°C for 50 min and terminated by heating to 70°C for 15 min and chilling on ice. One microliter of first strand cDNA mixture was used for PCR amplification in 50 μ l of 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 200 mM dNTP, 1 μ M of each primer and 2.0 U of Taq DNA polymerase (Takara, Shiga). Primer sequences were the following: for human cyclooxygenase-1 (hCOX-1) (24), sense primer (5'-TGC CCAGCTCCTGGCCCGCCGCTT-3') and antisense primer (5'-GTGCATCAACACAGGCGCCTCTTC-3'); for hCOX-2, sense primer (5'-TTCAAATGAGATTGT GGGAAAATTGCT-3') and antisense primer (5'-AGAT CATCTCTGGGCCTGAGTATCTT-3'); for hIL-6 (25), sense primer (5'-ATGAACTCCTTCTCCACAAGCG C-3') and antisense primer (GAAGAGCCCTCAGGCTC TGGACTG-3'); for β -actin, sense primer (5'-TGACGGG GTCACCCACACTGTGCCCATCTA-3') and antisense primer (5'-CTAGAAGCATTGCGGTGGACGATGGAG GG-3'). One cycle of PCR consisted of 94°C for 1.5 min (denaturing), 62°C for 1.5 min (annealing), and 72°C for 1.5 min (elongation). Twenty-five cycles for COX-1 and COX-2 and 35 cycles for IL-6 and β -actin were performed. The predicted lengths of the PCR products for each PCR are 305 bp, 303 bp, 628 bp and 662 bp, respectively. RT-PCR was semi-quantified by densitometrically scanning (GT-7000 ART; EPSON, Tokyo). For relative semi-quantitation, the densitometry value of each PCR-product was normalized for load based on β -actin levels. In addition, a linear correlation between RNA input and PCR product was examined.

Measurement of COX enzyme activity

We investigated the effects on COX-1 and COX-2 enzyme activity with reference to the method of Futaki et al. (26). Briefly, a COX reaction was conducted by incubation at 37°C for 2 min in a standard mixture (500 μ l) containing 100 mM Tris-HCl (pH 8.0), 1 mM hematin, 2 mM phenol, 10 U COX-1 or COX-2, and then [¹⁴C]arachidonic acid was added to the reaction mixture at 37°C for 2 min. The reaction was stopped by chilling on ice. To extract PGE₂, 2 ml of *n*-hexane:ethyl acetate (2:1 V/V) was added to the reaction mixture, and the preparation was centrifuged at 2,000 rpm for 5 min. The aqueous phase was frozen and the organic solvent phase was removed. PGE₂ extraction was performed twice. The amount of PGE₂, including the aqueous phases, was measured by radioimmunoassay. COX-1 (prostaglandin H synthases, Cayman Chemical Co.) and COX-2

(prostaglandin H synthases, Cayman Chemical Co.) were used.

Statistics

All data were represented the mean $\pm$ S.E.M. and analyzed by Dunnett's multiple comparison test. $P < 0.05$ was regarded to be statistically significant.

RESULTS

Effect on the production of TNF- α by RAW 264.7 cells

Mesoporphyrin at concentrations between 10⁻⁸ to 10⁻⁶ M had no effect on the production of TNF- α by RAW 264.7 cells (Fig. 1). Dexamethasone at a concentration of 10⁻⁵ M inhibited TNF- α production.

Effect on the production of IFN- γ by 1E10.H2 and IL-4 by D10.G4.1 cells

Mesoporphyrin at concentrations between 10⁻⁷ and 10⁻⁵ M inhibited the production of IFN- γ by 1E10.H2 cells in a concentration-related manner. On the contrary, mesoporphyrin had no effect on the production of IL-4 by D10.G4.1 cells (Fig. 2). The production of both cytokines was significantly inhibited by 10⁻⁶ M cyclosporin A, but not by 10⁻⁶ M indomethacin.

Fig. 1. Effect of mesoporphyrin and dexamethasone (Dex) on LPS-induced TNF- α production by RAW264.7 cells. Cells were incubated with LPS for 48 hr. Data are expressed as a percentage of the control values. Each column represents the mean $\pm$ S.E.M. of four experiments. Cell viability is not affected by each drug in the employed concentrations. **Statistically significant difference from the control at $P < 0.01$.

Fig. 2. Effect of mesoporphyrin and cyclosporin A (CyA) on Con A-induced IFN- γ production by 1E10.H2 cells (A) and Con A-induced IL-4 production by D10.G4.1 cells (B). Cells were incubated with Con A for 48 hr. Each column represents the mean $\pm$ S.E.M. of four experiments. *,**Statistically significant difference from the control at $P < 0.05$, $P < 0.01$, respectively.

Effect on the production of IL-6 and PGE₂ by MG-63 cells

Mesoporphyrin at concentrations between 10^{-7} and 10^{-5} M clearly inhibited the production of IL-6 and PGE₂ by MG-63 cells in a concentration-dependent manner (Fig. 3). Our previous preliminary data indicate that the production of IL-6 is a secondary event following the

generation of PGE₂, which resulted from COX-2 activation. To analyze the inhibitory mechanism, the effects of mesoporphyrin on the expression of COX-1, COX-2 and IL-6 mRNAs in MG-63 cells were investigated (Fig. 4). COX-1 mRNA, which is expressed constitutively, slightly increased after stimulation with IL-1 β . COX-2 and IL-6 mRNAs expression increased markedly after stimulation

Fig. 3. Effect of mesoporphyrin and prednisolone (Pred) on IL-1 β -induced PGE₂ (A) and IL-6 (B) production by MG-63 cells. Cells were incubated with IL-1 β for 48 hr (PGE₂) or 96 hr (IL-6). Each column represents the mean $\pm$ S.E.M. of four experiments. **Statistically significant difference from the control at $P < 0.01$.

with IL-1 β . Mesoporphyrin suppressed the expression of IL-6 mRNAs but not COX-1 or COX-2 mRNAs. Prednisolone slightly decreased the expression of COX-1 mRNA and suppressed the expression of COX-2 and IL-6 mRNA completely. Indomethacin and NS-398 suppressed the expression of IL-6 mRNA but not COX-1 or COX-2 mRNA. Next we examined the effect of mesoporphyrin on COX enzyme activity and compared it to the effects of prednisolone, NS-398 and indomethacin. Mesoporphyrin at concentrations between 10^{-8} and 10^{-4} M caused the inhibition of COX-2 enzyme activity in a concentration-dependent manner ($IC_{50}=7.87 \times 10^{-7}$ M) (Fig. 5). Moreover, mesoporphyrin inhibited COX-1 enzyme activity ($IC_{50}=2.75 \times 10^{-6}$ M). NS-398 inhibited COX-2 enzyme activity dose-dependently ($IC_{50}=2.82 \times 10^{-7}$ M) and indomethacin inhibited both COX-1 and COX-2 enzyme activity, the IC_{50} being 2.63×10^{-7} and 1.38×10^{-6} M, respectively. Prednisolone at a concentration of 10^{-4} M had no effect on COX-1 or COX-2 enzyme activity.

DISCUSSION

The present results indicate that mesoporphyrin in-

hibits IFN- γ production by Th1 cells and IL-6 production by osteoblast-like cells. Moreover, the inhibition of IL-6 production in osteoblast-like cells is mainly due to the inhibition of PGE $_2$ generation by interfering with the activities of COX-1 and -2. In addition, mesoporphyrin has no effect on the production of TNF- α by macrophage-like cells and IL-4 production by Th2 cells. These data indicate that the inhibition of cytokine production is one of the anti-inflammatory mechanisms of mesoporphyrin.

Many investigators have reported the pathophysiological role of cytokines in the development of immunological inflammation, especially RA. TNF- α , IFN- γ , IL-1 β and IL-6 are the most important cytokines as candidates for compounds involved in the pathological processes (27–30). Among these cytokines, TNF- α is demonstrated to be an important target molecule as a remedy for RA (31–36). In the present study, we have demonstrated no effect of mesoporphyrin on the production of TNF- α by macrophage-like RAW264.7 cells. This was confirmed by in vivo experiments using the LPS-induced TNF- α production system in mice pretreated with *Corynebacterium parvum* (H. Nagai et al., unpublished data). These results indicated that mesoporphyrin has no effect on the pro-

Fig. 4. Effect of mesoporphyrin on IL-1 β -induced COX-1, COX-2 and IL-6 mRNAs expression in MG-63 cells. MG-63 cells (2×10^5 cells) were stimulated with 0.5 ng/ml IL-1 β . After stimulation with IL-1 β for 12 hr, total RNA was extracted according to the manufacturer's instructions (Isogen). A: lane 1, IL-1 β (-); lane 2, IL-1 β (+); lane 3, IL-1 β + prednisolone; lane 4, IL-1 β + indomethacin; lane 5, IL-1 β + NS-398; lane 6, IL-1 β + mesoporphyrin; lane 7, positive control. Positive control was taken from human peripheral monocyte with stimulation of LPS for 6 hr. B: The intensities of COXI, COXII and IL-6 band intensities were semi-quantified. All PCR amplifications were performed at least twice with multiple sets of experimental RNAs. Pred, Meso and IND mean prednisolone, mesoporphyrin and indomethacin, respectively. ■: COX-1/ β -action, □: COX-2/ β -action, ▨: IL-6/ β -action.

duction of $\text{TNF-}\alpha$ by macrophages.

In the second part of the experiments, we have examined the effect of mesoporphyrin on the cytokine production by Th1 and Th2 cells. Many researchers have reported that both Th1 and Th2 cells play an important role for the development of inflammatory diseases including arthritis in humans and animal models (9–13, 20, 21). In the present experiments, mesoporphyrin inhibits the production of $\text{IFN-}\gamma$ by Th1 cells but not IL-4 by Th2 cells. As for the role of $\text{IFN-}\gamma$ in the arthritis, Williams et al. (37) and Bissier et al. (38) have reported that administration of anti- $\text{IFN-}\gamma$ monoclonal antibody suppressed the onset of collagen-induced arthritis in mice. In addition, Schultz-Koops et al. (39) reported that T cell clones from RA synovium and peripheral lymphocytes produced large amounts of $\text{IFN-}\gamma$ but not IL-4. These results indicate the importance of $\text{IFN-}\gamma$ in the development of arthritis. In our previous study, mesoporphyrin

inhibited CD4^+ T cell activation and the cell-mediated delayed type hypersensitivity in arthritic mice (6). In general, the cell mediated immune response is mediated by $\text{IFN-}\gamma$ through Th1 cell activation (36, 37). These results suggest that the inhibition of $\text{IFN-}\gamma$ production by interfering with the activation of Th1 cells may participate in the anti-inflammatory action of mesoporphyrin. In contrast, mesoporphyrin did not affect the production of IL-4 by Th2 cells. IL-4 is reported to play a role as an anti-inflammatory cytokine in the development of RA (27, 28). No influence on IL-4 production seems to be beneficial for the appearance of anti-inflammatory action of mesoporphyrin.

Regarding the role of IL-6, many possible roles in arthritis have been reported (40, 41). One of the important roles of IL-6 is an activation of osteoclast cells resulting in articular osteoporosis in RA patients (42). Sambrook et al. have demonstrated that progressive bone

Fig. 5. Effect of mesoporphyrin, indomethacin, NS-398 and prednisolone on COX-1 (○) and COX-2 (●) activity. Each point represents the mean of three experiments.

loss has occurred in RA patients (43), and the bone loss is mainly caused by activation of osteoclast by osteoblast-derived IL-6 (44). This is the reason why we examined the effect of mesoporphyrin on IL-1-induced IL-6 production by MG-63, human osteoblast-like cells. The results indicated that mesoporphyrin clearly inhibited the production of IL-6 by osteoblast-like cells.

Since IL-6 is well known to be one of the most important pathological cytokines in the development of the arthritis, we have examined the inhibitory mechanism of mesoporphyrin on IL-6 production by measuring PGE₂ production, COX mRNA expression and COX activity. As shown in Figs. 4 and 5, mesoporphyrin inhibited the expression of IL-6 mRNA and COX activities without affecting the expression of COX mRNAs. Indomethacin and NS-398 showed similar effects. Prednisolone inhibited IL-6 production, IL-6 mRNA expression and COX mRNA expressions. These data indicate that mesoporphyrin has a similar effect to NS-398 and indomethacin but not prednisolone. The concentration for 50% inhibition (IC₅₀) of COX-2 and COX-1 by mesoporphyrin was 7.87×10^{-7} M and 2.75×10^{-6} M, respectively. Mesoporphyrin showed a selective inhibition to COX-2 at low concentration range. These results suggest that mesoporphyrin inhibited the production of IL-6 by the inhibition of PGE₂ generation mainly through the interfering with COX-2 activity. Further detailed experiments will be necessary to determine the IL-1 β -induced IL-6 production in MG-63 cells and the mechanism of the inhibition of IL-6 production by mesoporphyrin.

Regarding the inhibitory mechanism of IFN- γ production by Th1 cells, mesoporphyrin shows a different mechanism from the inhibition of COX activities. There are some reports showing a low efficacy of indomethacin and other nonsteroidal anti-inflammatory drugs (NSAID) on Th1-cell-mediated delayed type hypersensitivity (45, 46). Moreover, as indicated in Fig. 2, indomethacin did not affect the Th1 activity in terms of the production of IFN- γ . These data indicate that the anti-inflammatory mechanism underlying the inhibition of IFN- γ production is different from the mechanism for the inhibition of IL-6 production. Further experiments will be necessary to elucidate the inhibitory mechanism of IFN- γ production by mesoporphyrin.

In conclusion, the present data indicate that mesoporphyrin inhibits IFN- γ and IL-6 production but not TNF- α production. In addition, the inhibition of IL-6 production is related to the inhibitions of PGE₂ production and COX activity. These results indicate that the inhibition of cytokine production is one of the important anti-inflammatory actions of mesoporphyrin that is involved in the inhibition of experimental arthritis by this drug.

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