Clofazimine (B663) is a phenazine iminoquinone derivative, specifically, a riminophenazine dye with the empirical formula C_{27}H_{22}Cl_{12}. In the 1950s, Barry et al. synthesized a large number of compounds by progressive chemical alteration of the anilinoaposafranine molecule, several of which showed antimycobacterial activity both in vitro and in experimental animals (1). Of these compounds, clofazimine (or Lamprene or B663) was found to be highly active against mycobacteria with the least toxicity. Chang (4) observed the antibacterial activity of clofazimine and in experimental animals (1). Of these compounds, clofazimine (or Lamprene or B663) was found to be highly active against mycobacteria with the least toxicity. Chang (4) observed the antibacterial activity of clofazimine against Mycobacterium leprae at about the same time as its anti-M. lepra activity was reported by Browne (2) and Browne and Hogendoorn (3). Later, after the introduction of the mouse footpad method of Shepard and Chang (22), its antibacterial activity against M. lepra was demonstrated (18).

Clofazimine has bifunctional activity: antibacterial and anti-inflammatory. It was used in the treatment of leprosy for its antibacterial action against M. lepra. Later, it was also found to possess an anti-inflammatory action which makes it a very useful drug in the treatment of acute reactions, including erythema nodosum leprosum (ENL), neuritis, iritis, etc., although its mechanism of action is unknown (2). In vitro studies on the effect of clofazimine on immune cells have been conducted. Clofazimine increases superoxide anion production and degranulation by stimulated neutrophils. In vivo studies on the mechanism underlying this pro-oxidative effect seem to involve stimulation of phospholipase A2 (PLA2) activity with subsequent accumulation of arachidonic acid and lysophospholipids, which act as second messengers to activate oxidase (10). In addition, a number of reports have demonstrated the effects of clofazimine that might predict increased immune activity. Lysosomal enzyme activity of cultured macrophages was upregulated by clofazimine (21). Peripheral blood monocytes from healthy volunteers have been demonstrated to exhibit increased major histocompatibility complex II expression following incubation with clofazimine (25). Increased oxygen uptake during phagocytosis was observed in neutrophils derived from patients with pyoderma gangrenosum during clofazimine therapy (5). Suppressor T-cell activity was decreased in mycobacteria-infected mice during clofazimine treatment (26). However, the mechanisms underlying the anti-inflammatory action of clofazimine are still unclear.

In the present study, we examined the effect of clofazimine on macrophages and found that the drug possessed apoptosis-inducing activity.

**MATERIALS AND METHODS**

Drug and chemicals. Clofazimine (Sigma-Aldrich Co., St. Louis, MO), rifampin (catalog no. R3501; Sigma-Aldrich Co.), and dapsone (DDS; Biomol Research Inc., Butler Pike Plymouth Meeting, PA) were dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C until use. Ampicillin was obtained from Sigma-Aldrich Co.

Culture of human macrophages and isolation of bacilli. Human peripheral blood was obtained under informed consent from healthy individuals. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom) gradient centrifugation (12). The cells were suspended in AIM-V medium (Gibco BRL, Invitrogen Corp., Carlsbad, CA), and 1 × 10^6 PBMCs were cultured in a well of a 24-well tissue culture plate (Falcon; Becton Dickinson Labware, Becton Dickinson and Company, Franklin Lakes, NJ) containing 15-mm round coverslips (Nunc Thermanox coverslips; Nalge Nunc, Thermo Scientific, Rochester, NY) at 37°C in a 5% CO2 incubator for adherence of monocytes. After 1 h incubation, the coverslips were washed with Hank’s balanced salt solution (HBSS; Sigma-Aldrich Co.) to remove nonadherent cells. The monocytes on the coverslips were cultured in a new 24-well plate containing RPMI 1640 medium (Sigma-Aldrich Co.) supplemented with 25 mM HEPES, 10% fetal bovine serum (FBS; Bio Whittaker Co., Walkersville, MD), 2 mM L-glutamine, and 100 μg/ml ampicillin (RPMI-10F) in the presence of 40 ng/ml of granulocyte-macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN). After 10 days, the cells were differentiated into macrophages and used for experiments. In some experiments, PBMCs were cultured in 35-mm cell culture dishes (Corning Inc., Corning, NY) for adherence, and adherent monocytes were cultured for 10 days. Human monocyctic leukemia cell line THP-1 was maintained in RPMI 1640 medium containing 15% fetal bovine serum.

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**M. leprae** (Thai-53 strain) was isolated from the footpads of BALB/c nu/nu mice that had been inoculated with **M. leprae** 8 months prior to isolation, and the bacillary number was enumerated according to the method of Shepard and Chang (22).

**Light and phase-contrast microscopy.** Macrophages on the coverslip were fixed with absolute methanol, followed by performing Giemsa stain (Wako Co., Japan). After they were mounted on a glass slide, the cells were observed under a light microscope (Optiphot-2; Nikon Co., Tokyo, Japan). Photographs were taken with a digital camera (Nikon F70s). Macrophages in 35-mm dishes were incubated in the presence of clofazimine and subsequently observed under a phase-contrast microscope (Olympus CX41 with ×10- and ×20-objective lenses). Photographs were taken with an Olympus DPS50 system. Image acquisition and data processing were done using the DP controller software.

**Fluorescence microscopy.** Fluorescence staining for DNA was employed. Macrophages were cultured in an 8-well chamber slide (Lab-Tek II chamber slide system; Nalg Nunc). The cells were incubated in the presence of clofazimine and subsequently fixed with 2.5% glutaraldehyde in phosphate-buffered saline (PBS), Hoechst 33342 dye (Sigma-Aldrich Co.) in PBS was added to the wells at a final concentration of 10 μM, and the slide was incubated for 1 h at 37°C. The cells on the slide were observed under a fluorescence microscope (Olympus IX60 with ×40-objective lens) equipped with an Olympus DPS50 system. The digital images were processed with DP controller software.

**Determination of cell death.** Cell viability was determined using the colorimetric method (Cell Titer 96 Aquo nonradioactive cell proliferation assay; Promega Corp., Madison, WI). Briefly, cells in a 24-well plate were incubated in the presence of clofazimine in phenol red-free RPMI 1640 medium containing 10% FBS, followed by addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (Wako Pure Chemical Industries). After 1 h incubation at 37°C, an aliquot of medium was transferred into a well of a 96-well plate, and the color developed was measured by a spectrophotometer at 490 nm. In addition, the activity of lactate dehydrogenase (LDH), released from dead cells into culture supernatants, was measured by a colorimetric assay (Cytotox 96 nonradioactive cytotoxicity assay; Promega Corp.). The color that developed in a sample incubated with LDH substrate was measured by a microplate reader at 490 nm. The color that developed in a sample incubated with a substrate for caspase-3, Asp-Glu-Val-Asp-g-nitroanilide, was added to the tube. After incubation for 2 h at 37°C, the samples were transferred into a well of a 384-well plate and read by a plate reader at 405 nm (Infinite F200; Tecan Systems Inc., San Jose, CA). The background reading was obtained by subtracting the reading for the reaction buffer from the reading for the lysate samples.

**RESULTS**

**Morphological changes observed after treatment with clofazimine.** Macrophages differentiated from human monocytes were incubated in the presence of 10 μg/ml of clofazimine for 20 h. The change in cell morphology was observed under a phase-contrast microscope. As shown in Fig. 1B, in the presence of clofazimine, the cells exhibited shrinkage in cell size and membrane blebbing. The death of more than 80% of cells was observed (Fig. 1B). As a control, Fig. 1A shows the normal morphology of macrophages. By Giemsa stain, too, these clofazimine-treated cells exhibited shrinkage in cell size, accompanied by the appearance of fragmented smaller nuclei (arrow in Fig. 1D), suggesting the apoptotic nature of the cells. Non-treated macrophages showed intact nuclei (arrow in Fig. 1C).

Again, the change of nuclear structure was confirmed by Hoechst dye staining. Under a fluorescence microscope, nuclear condensation and membrane blebbing were observed in the clofazimine-treated cells (Fig. 1F and G), in contrast to normal cells, which showed intact nuclei (Fig. 1E). Similar fragmentation or condensation of chromatin was observed in THP-1 cells (data not shown). Such morphological changes were not observed in THP-1 cells treated with rifampin or dapsone at a concentration up to 50 μg/ml. Also, DMSO, which was used as a solvent for clofazimine at a concentration of 0.2%, had no effect on cell morphology or cell functions (negative control).

**Cell death-inducing activity of clofazimine determined by colorimetric assay.** Cell death was determined by a biochemical analysis using a colorimetric method. The conversion of the tetrazolium compound into soluble formazan is accomplished by metabolically active cells. When higher concentrations up to 10 μg/ml of clofazimine were employed in macrophage cultures, decreased color intensity of soluble formazan was observed, indicating cell death (Fig. 2A). Cell death was also observed in THP-1 cells (Fig. 2B). Hansen’s disease is caused by infection of macrophages with **M. leprae**; therefore, we are curious to know whether **M. leprae** infection affects the cell death-inducing activity of clofazimine. When we infected macrophages with **M. leprae** at a multiplicity of infection (MOI) of 10 or 30, we found no significant difference in the induction of cell death in the presence of 10 μg/ml clofazimine, indicating that the bacilli did not inhibit or enhance clofazimine-induced cell death (Fig. 2C). Another method of determining cell death is by measurement of LDH release from...
dead cells. As shown in Fig. 3, more LDH release was observed in the manner dependent on the concentration of clofazimine.

Clofazimine treatment induces DNA ladder formation in macrophages. We examined the condition of DNA in clofazimine-treated THP-1 cells. Agarose gel electrophoresis showed fragmentation of DNA into integer multiples of 180 bp, a so-called DNA ladder (Fig. 4A), suggesting that DNA endonuclease was activated by clofazimine treatment. Therefore, we examined the effect of one of the apoptosis inhibitors, ZnCl2, which is known to possess suppressing activity for endonuclease, and found that clofazimine-induced DNA fragmentation in THP-1 cells was completely blocked by ZnCl2 treatment even at a low concentration of 0.25 mM ZnCl2 (Fig. 4B), although it is still not clear whether ZnCl2 can directly block the activity of clofazimine. Moreover, it was evident that neither cell death nor DNA fragmentation was induced by other antileprosy drugs, such as DDS or rifampin (Fig. 4C).

Clofazimine-induced cell death is mediated by activation of caspase-3. Caspases are known to be central regulators of apoptotic cell death, and caspase-3, which locates downstream of the caspase pathway, is one of the key executioners of apoptosis. Upon apoptotic stimulation, caspases are cleaved into active fragments. Figure 5 shows a Western blot analysis of extracts from THP-1 cells and macrophages cultured in the presence of clofazimine. Enhanced expression of cleaved caspase-3 was detected in cells (Fig. 5A and B). In addition, caspase-9 was also cleaved. A DNA-repairing enzyme, PARP, which is cleaved by caspase-3, was significantly activated in clofazimine-treated THP-1 cells (Fig. 5A). We next measured the caspase activity by colorimetric assay (Fig. 5C). The induction of caspase-3 by clofazimine was significantly high in macrophages as well as THP-1 cells.

Clofazimine enhanced PGE2 production in M. leprae-infected macrophages. Monocyte-derived macrophages were preincubated in the presence of clofazimine for 4 h, followed by replenishment with M. leprae-containing medium for 20 h. The culture supernatants were collected, and the PGE2 concentration was measured. As shown in Fig. 6, clofazimine clearly enhanced PGE2 production in macrophages.

DISCUSSION

Riminophenazines are structurally phenazine compounds which were derived from lichens historically and were targeted for treatment of tuberculosis. The first clinically developed phenazine compound was clofazimine, whose activity has been extended to other mycobacterial diseases (1, 17). In test animals, the drug was found to inhibit the growth of mycobacteria in vivo, as well as in vitro (22), but the molecular mechanism of clofazimine in inducing anti-M. leprae activity is still not yet clear.

In the present study, it was found that both human monocyte-derived macrophages and THP-1 cells exhibited marked decreases in their metabolic activity in the presence of 10 μg/ml clofazimine. Under a phase-contrast microscope, 80% of the cells showed irregular morphology with shrinking in cell size, and by a precise time course study, it was revealed that the morphological changes were evident from 6 h incubation with clofazimine. From this early time point, the cell body began to shrink, accompanied by membrane blebbing, which was also
evident from Giemsa stain and Hoechst staining of the nuclei (Fig. 1). Interestingly, the dose of clofazimine (10/μg/ml) required to cause cell death was equivalent to the dose required to exhibit anti-\textit{M. leprae} activity in vitro by radiorespirometry (data not shown), the dose of which is in concordance with the dose required to kill \textit{M. leprae} reported by Franzblau and O’Sullivan (7). Moreover, in our study, at 5-μg/ml concentrations of clofazimine, \textit{M. leprae} viability was lowered in in vitro experiments with \textit{M. leprae}-infected macrophages, and with this dose, \textit{M. leprae} was found not to inhibit clofazimine-induced cell death. Therefore, clofazimine might inhibit mycobacterial growth through an alternative way by inducing apoptosis of host cells. Although the concentration of clofazimine in sera of patients taking regular doses of the drug is as low as 1 to 2 μg/ml, fat-soluble clofazimine readily accumulates in cells. In one patient, 7 months treatment with clofazimine (200 mg/day) resulted in accumulation of needle-shaped crystal inclusions in his alveolar macrophages (20). In another report, clofazimine-induced crystal-storing histiocytosis was observed in a leprosy patient (23). So, we are of the opinion that in some cells, the concentration of clofazimine is higher (10 to 20 μg/ml) than in others, so we have used a concentration of 10 μg/ml for our experiments.

Normally, cells undergo distinct morphological changes when they progress through either necrosis or apoptosis. Necrosis occurs when cells are exposed to an extreme variance from physiological conditions, resulting in damage to the plasma membrane. As such, necrosis is characterized by cell swelling and disruption of cellular organelles, with little change in the chromatin initially. In contrast, apoptotic cells shrink in size, undergo membrane blebbing, and exhibit marked alterations in their chromatin structure at an early stage under normal physiological conditions. As mentioned earlier, treatment with clofazimine resulted in highly condensed chromat in the nucleus and membrane blebbing, indicating macrophages undergoing apoptosis. To confirm this, DNA from clofazimine-treated THP-1 cells was examined. Fragmented DNA was

FIG. 2. Clofazimine-induced cell death in macrophages and THP-1 cells. Human monocyte-derived macrophages (A) and THP-1 cells (B) were incubated with various concentrations of clofazimine for 24 h, followed by determination of viable cells by the Cell Titer 96 cell proliferation assay. The results are representative of three independent cell culture tests. The cell death-inducing effect of clofazimine in the presence of \textit{M. leprae} was also examined. Monocyte-derived macrophages were infected with \textit{M. leprae} at an MOI of 10 or 30 per cell for 24 h. The infected cells were further incubated with 10 μg/ml clofazimine for another 24 h, followed by determination of viable cells by Cell Titer 96 cell proliferation assay (C). The results are representative of three independent cell culture tests. OD, optical density.

FIG. 3. LDH release from clofazimine-treated macrophages. Human monocyte-derived macrophages were incubated in the presence of the indicated concentrations of clofazimine for 24 h, and the LDH activity was measured. The results were obtained from triplicate cultures and are representative of three independent cell culture tests. OD, optical density.
demonstrated, suggesting that DNA endonuclease was activated causing apoptosis.

We observed that _M. leprae_ by itself does not induce apoptosis of human cells. Similarly, infection of mouse macrophages with viable _M. leprae_ was shown not to induce apoptosis (11). Although apoptosis is induced when macrophages infected with _M. leprae_ are treated with clofazimine, the host cell viability does not change significantly in the presence of _M. leprae_. Nevertheless, the viability of _M. leprae_ in macrophages was significantly lower in clofazimine-treated cells than infected cells not treated with clofazimine (data not shown). Therefore, we can speculate that clofazimine induces apoptosis of _M. leprae_-infected macrophages, which in turn inhibits _M. leprae_ growth.

Consequently, we investigated the pathways involved in the execution of macrophage apoptosis (6, 14). We observed enhanced expression of cleaved caspase-3, caspase-9, and PARP by Western blotting (A). Similarly, monocye-derived macrophages were incubated in the presence of 10 μg/ml of clofazimine, and the cell lysates were examined for cleaved caspase-3 and caspase-9 expression (B). The caspase activity in clofazimine-treated macrophages and THP-1 cells was analyzed. Macrophages were incubated in the presence of 10 μg/ml of clofazimine for 6 h, and the caspase-3 activity in the cell lysates was determined by colorimetric assay (C). The results are representative of three independent cell culture tests.

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is known to have a favorable influence on the reversal reaction in human leprosy (2). Browne and Hogenzeil found that clofazimine controlled persistent exacerbations in patients who were corticosteroid dependent for controlling the passing reactions, including ENL, and suggested that the drug may exert a suppressive effect on the development of acute exacerbation in lepromatous leprosy (3). These observations have been followed by those of later workers, and one of the special indications for use of clofazimine is the presence of acute reactions or a chronic recurrent reaction in lepromatous leprosy. These clinical data suggest that the mechanisms underlying the action of the drug in these leprosy patients mainly seem to be anti-inflammatory, although there is no direct evidence. Macrophages are capable of elaborating a series of biochemical products with potent immunomodulatory activities. We have observed enhancement of the production of PGE$_2$ when macrophages were pretreated with clofazimine. PGE$_2$ is released from arachidonic acid by PLA2. The enzyme is reported to be stimulated in clofazimine-treated neutrophils (10). With respect to B-cell function, prostaglandins of the E series (PGE) inhibit both B-cell proliferation and the generation of antibody-forming cells, and also, B-cell tolerance is induced by PGE$_2$ (9, 24). T-cell proliferation is also suppressed by PGE$_2$ (13). The reaction to leprosy involves antibody (ENL caused by immune complex) and cells (delayed-type hypersensitivity mediated mainly by T cells). The mechanisms underlying the immunomodulatory role of clofazimine are still not clear, but the present study clarifies certain aspects. Apoptosis induced in macrophages might explain the anti-inflammatory activities of clofazimine in vivo.

In conclusion, our findings suggest that clofazimine induced apoptosis of macrophages through the activation of caspases. The data indicate that the action of clofazimine in leprosy patients may be at least partially mediated by apoptosis.

FIG. 6. Enhancement of PGE$_2$ production by clofazimine in M. leprae-infected macrophages. Macrophages were preincubated in the presence of the indicated doses of clofazimine for 4 h, followed by infection with M. leprae ($5 \times 10^7$/well), and the culture was continued for another 20 h. The amount of PGE$_2$ in the culture supernatants was measured. The results are representative of three independent cell culture tests.

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REFERENCES


