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Chloroquine inhibits production of TNF-α, IL-1β and IL-6 from lipopolysaccharide-stimulated human monocytes/macrophages by different modes

C.-H. Jang, J.-H. Choi, M.-S. Byun and D.-M. Jue

Objectives. **TNF-α, IL-1 and IL-6 are known to have primary roles in the pathogenesis of rheumatoid arthritis and other inflammatory diseases. The anti-rheumatic drug chloroquine has been shown to inhibit TNF-α, IL-1 and IL-6 production from mononuclear phagocytes. We examined the underlying mechanisms involved in the chloroquine-induced inhibition of cytokine production.**

Methods. **Human peripheral blood mononuclear cells and monocytes/macrophages and monocytic U-937 and THP-1 cells were stimulated with lipopolysaccharide, and TNF-α, IL-1β and IL-6 production was measured by ELISA. Levels of mRNA were measured by northern blotting and reverse transcription–polymerase chain reaction. Synthesis of 26-kDa TNF-α precursor was measured by metabolic labelling and immunoprecipitation analysis. Transcription rate was determined by nuclear run-on assay.**

Results. **TNF-α release from the cells was inhibited by chloroquine, whereas the steady-state level of TNF-α mRNA and synthesis of 26-kDa TNF-α precursor were not changed by chloroquine. In contrast, chloroquine-induced inhibition of IL-1β and IL-6 release was accompanied by a decrease in their steady-state mRNA levels. The transcription rates of the IL-1β and IL-6 genes were not changed by chloroquine, whereas the stability of IL-1β and IL-6 mRNA was decreased by chloroquine. Weak-base amines such as methylamine and ammonium chloride had no effect on the production of TNF-α, whereas they partially blocked the production of IL-1β and IL-6.**

Conclusions. **Our results indicate that chloroquine-mediated inhibition of TNF-α, IL-1β and IL-6 synthesis occurs through different modes in lipopolysaccharide-stimulated human monocytes/macrophages: it blocks the conversion of cell-associated TNF-α precursor to mature soluble protein, whereas it reduces the levels of IL-1β and IL-6 mRNA, at least in part, by decreasing their stability and by a pH-dependent mechanism.**

KEY WORDS: Chloroquine, Rheumatoid arthritis, Cytokines, Gene regulation, Monocytes/macrophages, Inflammation.

Prolonged production of proinflammatory cytokines such as TNF-α, IL-1 and IL-6 has been shown to play a pivotal role in synovial cell activation and joint destruction in rheumatoid arthritis (RA) [1, 2]. TNF-α produced mainly by monocytes and macrophages exerts diverse effects in RA pathogenesis by stimulating other cells and inducing tissue damage [3]. In stimulated cells, TNF-α is synthesized as a membrane-associated 26-kDa precursor that is then proteolytically cleaved to release soluble, mature 17-kDa protein into the medium [4, 5]. IL-1 produced mainly by monocytes and macrophages in the synovial tissue of RA patients has been strongly implicated in the joint damage [6]. IL-6 is a pleiotropic cytokine produced by T cells, macrophages and synovial fibroblasts in inflammatory joint tissues [7]. IL-6 has been known to promote synovitis by stimulating antibody production as a result of its effect on B-cell maturation, and also by activating T cells, and inducing proliferation of synovial fibroblasts [8]. Administration of agents blocking actions of these cytokines resulted in dramatic remission of arthritic symptoms and reduction in inflammation markers in patients with long-standing RA [9–12].

Antimalarial drugs such as chloroquine and hydroxychloroquine are widely used in the treatment of RA because they are relatively well tolerated in comparison with other anti-rheumatic agents of similar potency [13, 14]. Chloroquine was shown to block TNF-α and IL-6 synthesis in lipopolysaccharide (LPS)-stimulated mouse macrophages and human monocytes [15–17], although the inhibitory mode observed was different between mouse and human cells. In our previous study with mouse macrophage RAW 264.7 cells, chloroquine was shown to inhibit TNF-α synthesis mainly by blocking conversion of cell-associated 26-kDa TNF-α precursor to the soluble 17-kDa mature form, rather than by inhibiting induction of TNF-α mRNA or synthesis of 26-kDa TNF-α precursor [18]. However, other studies with human peripheral blood mononuclear cells (PBMCs) showed that chloroquine reduced LPS-induced expression of TNF-α, IL-1β and IL-6 mRNA, and cell-associated TNF-α [19, 20]. Chloroquine was shown to inhibit LPS-induced activation of extracellular signal-regulated kinase (ERK) 1/2 in human PBMCs and the expression of the TNF-α promoter-driven reporter gene in human monocytic THP-1 cells, suggesting that chloroquine blocks transcription of the TNF-α gene by interfering in LPS-induced activation of the ERK1/2 signalling pathway [21]. The chloroquine-induced decrease in IL-1β and IL-6 mRNA has not been studied in detail, and the underlying mechanism is unknown.

Here we studied the effect of chloroquine on the synthesis of TNF-α, IL-1β and IL-6 in LPS-stimulated human PBMCs and

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monocytes/macrophages, and also in monocytic THP-1 and U-937 cells. Our results show that chloroquine inhibits TNF-α release, but does not change the level of TNF-α mRNA or the synthesis of 26-kDa TNF-α precursor. We also observed that chloroquine inhibits IL-1β and IL-6 production by decreasing their mRNA levels, which is in turn caused by a decrease in mRNA stability rather than a change in transcriptional activity.

Materials and methods

Materials

Chloroquine (diphosphate salt), phorbol myristate acetate (PMA), ammonium chloride, methylamine, leupeptin and LPS (*Escherichia coli* 0127:B8) were purchased from Sigma Chemical (St Louis, MO, USA). Hydroxychloroquine sulphate (purity 99.3%; IntaPort Company, Ridgewood, NJ, USA) was a gift from Yuhan Industrial (Seoul, Korea). Mouse and rat monoclonal antibodies for enzyme-linked immunosorbent assay (ELISA) of human TNF-α and IL-6, fluorescein isothiocyanate (FITC)-labelled anti-TNF-α and anti-IL-6 antibodies, phycoerythrin (PE)-labelled antibody to CD14 were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). Monoclonal antibodies for ELISA of human IL-1β and recombinant human IL-1β and IL-6 were obtained from Pierce Biotechnology (Rockford, IL, USA). Recombinant human TNF-α was prepared by expression in *E. coli* as described previously [22]. Polyclonal antisera to human TNF-α were prepared by immunizing rabbits with purified recombinant protein.

Cell culture

Human leucocyte concentrate was obtained from the Korean Red Cross Blood Bank (Seoul, Korea). Each leucocyte concentrate was prepared from the blood of single donor.

PBMCs, were prepared from the leucocyte concentrate by Ficoll–hypaque density gradient centrifugation and monocytes/macrophages were isolated from PBMCs, by adherence to culture dishes in serum-free Dulbecco's modified Eagle's medium [23]. The adherent cells were recovered by scraping and resuspended in RPMI 1640 medium containing 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA). Human monocytic leukaemia THP-1 cells and histiocytic lymphoma U-937 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 containing 10% FBS. THP-1 and U-937 cells were incubated with PMA (20 ng/ml) for 48 h to induce differentiation to monocytes/macrophages before LPS stimulation [24]. Cells were incubated with chloroquine for 2 h before stimulation with LPS (1 μg/ml). Cell viability was measured by staining cells with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [25].

ELISA

TNF-α, IL-1β and IL-6 in the culture supernatant and the cell lysate were measured by sandwich ELISA, using specific monoclonal antibodies according to the manufacturer's instructions. To determine the level of cell-associated cytokines, cells were washed with phosphate-buffered saline (PBS) and lysed in a buffer containing 0.5% NP-40 [23]. Standard recombinant proteins were also diluted in PBS containing the same concentrations of NP-40 as the cell lysate. The amounts of cytokines in the culture supernatant and the cell lysate are presented as those produced from 1×10^6 cells.

Analysis of mRNA

mRNA of TNF-α, IL-1β and IL-6 was measured by northern blot analysis or reverse transcription–polymerase chain reaction (RT-PCR) after total RNA had been isolated from cells using an RNA isolation kit (RNA-Stat-60; Tel-Test, Friendswood, TX, USA). To measure mRNA by northern blot analysis, RNA samples (10–15 μg) were electrophoresed in an 1% agarose gel, transferred to nylon membrane, and hybridized with digoxigenin-labelled cDNA probes [18]. Blots were visualized by using anti-digoxigenin antibody and chemiluminescence detection (Roche Applied Science, Mannheim, Germany). Probes were prepared by PCR amplification of the respective cDNA with digoxigenin-11-dUTP. cDNA clones of human TNF-α and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were prepared as described previously [22, 23]. cDNAs of IL-1β and IL-6 were generated by RT-PCR with specific primer sets and template RNA obtained from U-937 cells and PMA- and ionomycin-stimulated human T cells, respectively. PCR-amplified cDNAs were cloned in pGEM-T vector (Promega, Madison, WI, USA). The primers for IL-1β cDNA were AGCCATGGCAGAAGTACCT and CAGC TCTCTTTAGGAAGACAC; those for IL-6 cDNA were ATGAACTCCTTCTCCACAAGCGC and GAAGAGCCCT CAGGCTGGACTG. To measure mRNA by RT-PCR, cDNA was synthesized using Moloney murine leukaemia virus reverse transcriptase (Promega) according to the manufacturer's instructions. PCR primers used were as described previously for β-actin, IL-1β and IL-6 [26]. The primers for TNF-α cDNA were TCTCGAACCCCGAGTGACAA and TGAAGAGGACCTG GGAGTAG. PCR amplification consisted of 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. All PCR were performed on various amounts of cDNA and cycle numbers to ensure that the products measured were in the linear range. Amplified products were separated on 1.5% agarose gels and stained with ethidium bromide, and band intensity was measured in a still video system (Eagle Eye II; Stratagene, La Jolla, CA, USA).

Flow cytometry

PBMCs treated with various agents were harvested, incubated for 20 min in PBS containing 10% normal human serum and 0.1% NaN_3, and washed in PBS containing 1% FBS and 0.1% NaN_3 (staining buffer). CD14 on the surface of monocytes/macrophages was stained with PE-conjugated monoclonal antibody for 30 min. Cells were washed in staining buffer, resuspended in fixation/permeabilization buffer (Cytofix/Cytoperm; BD Pharmingen), and incubated for 20 min. Cells were stained with FITC-labelled anti-TNF-α or anti-IL-6 monoclonal antibodies on ice for 30 min, washed with staining buffer, and analysed with a flow cytometer (FACSorter; Becton Dickinson, Mountain View, CA, USA).

Metabolic labelling and immunoprecipitation

THP-1 cells grown in a six-well plate (5×10^5 cells/ml) were incubated with PMA for 48 h and stimulated with LPS for 2 h in the absence or presence of chloroquine. The cells were incubated for 30 min in methionine/cysteine-deficient RPMI 1640 medium (ICN, Irvine, CA, USA) and labelled for another 30 min by adding [^{35}S]methionine/cysteine mix (NEN, Boston, MA, USA) (100 μCi/well). LPS and chloroquine were added to the starvation and labelling medium if necessary. Immunoprecipitation analysis of TNF-α precursor in the cell lysate was done with polyclonal anti-human TNF-α antibody as described previously [18]. After separation of immune complexes by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), the gel was fixed in 10% acetic acid/30% methanol, soaked in scintillant

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solution (22% 2,5-diphenyloxazole in acetic acid) for 1 h, dried, and exposed to X-ray film at –70°C.

Nuclear run-on assay

The nuclear run-on assay was performed as described previously [27] with some modifications. Isolated nuclei from monocytes/macrophages (1×10^7 cells) were incubated in the presence of [α-^{32}P]UTP, ATP, CTP and GTP at 37°C for 30 min. The synthesized transcript was isolated using RNA-Stat-60 and hybridized to nylon membrane (Schleicher & Schuell, Dassel, Germany) with immobilized spots containing 5 μg of linearized TNF-α, IL-1β, IL-6 and β-actin cDNA cloned in pGEM-T vectors. The hybridization was carried out at 50°C for 3 days and blots were washed twice in 2 × standard saline citrate (SSC), 0.1% SDS at 65°C for 30 min, once in 2 × SSC, RNase A (10 μg/ml) at 37°C for 60 min, and twice in 0.5 × SSC, 0.1% SDS at 37°C for 30 min before exposure to X-ray film or a phosphor imager plate (BAS-2500, Fujifilm, Osaka, Japan).

Results

Effect of chloroquine on the synthesis of TNF-α

To examine the effect of chloroquine on TNF-α synthesis, we determined TNF-α and intermediates of its synthesis in chloroquine-treated cells. First, we measured TNF-α production from various types of human cells, such as PBMCs, monocytes/macrophages and differentiated U937 and THP-1 cells. After stimulation of cells with LPS, we measured TNF-α released into the culture medium and that associated with the cells by ELISA (Fig. 1A). TNF-α production was negligible in non-stimulated cells, whereas addition of LPS induced a remarkable increase of its level. Incubation of cells with chloroquine resulted in a dose-dependent suppression of TNF-α production, resulting in >50% inhibition at 100 μM of chloroquine. The cell viability was >90% after incubation with 100 μM chloroquine (data not shown). Measurement of TNF-α in the cell lysate revealed that chloroquine did not change, or slightly increased, cell-associated TNF-α in PBMCs, monocytes (not evident in Fig. 1A because of the high level of TNF-α in the medium), U937 and THP-1 cells (Fig. 1A). We then analysed TNF-α mRNA by northern blotting in the same cells to test whether the inhibition of TNF-α production by chloroquine was caused by a decrease in its mRNA (Fig. 1B). In contrast to its inhibitory effect on TNF-α production, the steady-state level of TNF-α mRNA was not significantly decreased by chloroquine.

Because the above results suggested that the inhibitory effect of chloroquine on TNF-α synthesis appears at a post-transcriptional step, we then tested the effect of chloroquine on the synthesis of cell-associated 26-kDa TNF-α precursor. To confirm our results obtained by ELISA of cell lysate, we measured cell-associated TNF-α in monocytes/macrophages by flow cytometry using FITC-labelled anti-TNF-α antibody (Fig. 2A). LPS-induced expression of cell-associated TNF-α was not changed by chloroquine even at 100 μM concentration, while the expression of cell-associated IL-6 was remarkably reduced even by 25 μM chloroquine. We then examined the effect of chloroquine on the synthesis of membrane-associated TNF-α precursor by metabolically labelling LPS-stimulated PBMCs with [^{35}S]methionine/cysteine for 30 min in the absence or presence of chloroquine. The amount of TNF-α precursor synthesized was monitored by immunoprecipitation analysis of cell lysate with anti-TNF-α antibody and fluorography. As shown in Fig. 2B, the amount of radiolabelled 26-kDa TNF-α precursor was not reduced in chloroquine-treated cells compared with that in control cells incubated with LPS alone.

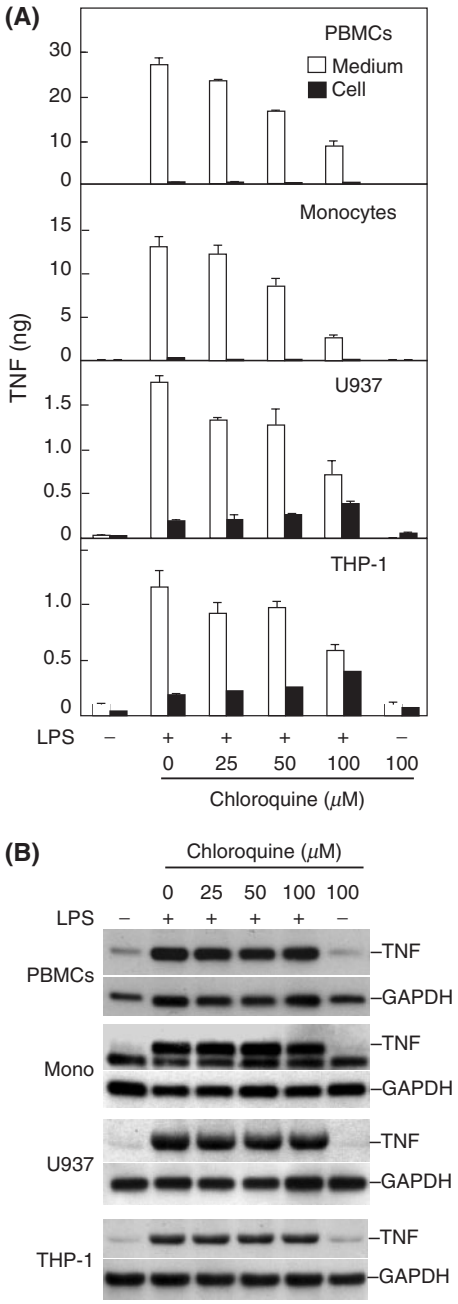

FIG. 1. Inhibition of TNF-α synthesis by chloroquine in the absence of concomitant decrease in TNF-α mRNA. (A) Human PBMCs, monocytes/macrophages, U937 and THP-1 cells were incubated with or without various doses of chloroquine for 2 h. After addition of LPS (1 μg/ml), PBMCs and monocytes/macrophages were incubated for 12 h, and U937 and THP-1 cells for 6 h. TNF-α in the culture medium and cell lysate was measured by ELISA. (B) Total cellular RNA was prepared from cells incubated as in (A) after 4 h of LPS induction. Northern blot analysis of TNF-α and GAPDH mRNA was carried out with digoxigenin-labelled probes and the bands were visualized by the chemiluminescence reaction. The data represent three independent experiments.

Effect of chloroquine on the synthesis of IL-1β and IL-6

To examine whether chloroquine inhibits the synthesis of other proinflammatory cytokines in a similar way to TNF-α, we measured the amounts of IL-1β and IL-6 released in the culture

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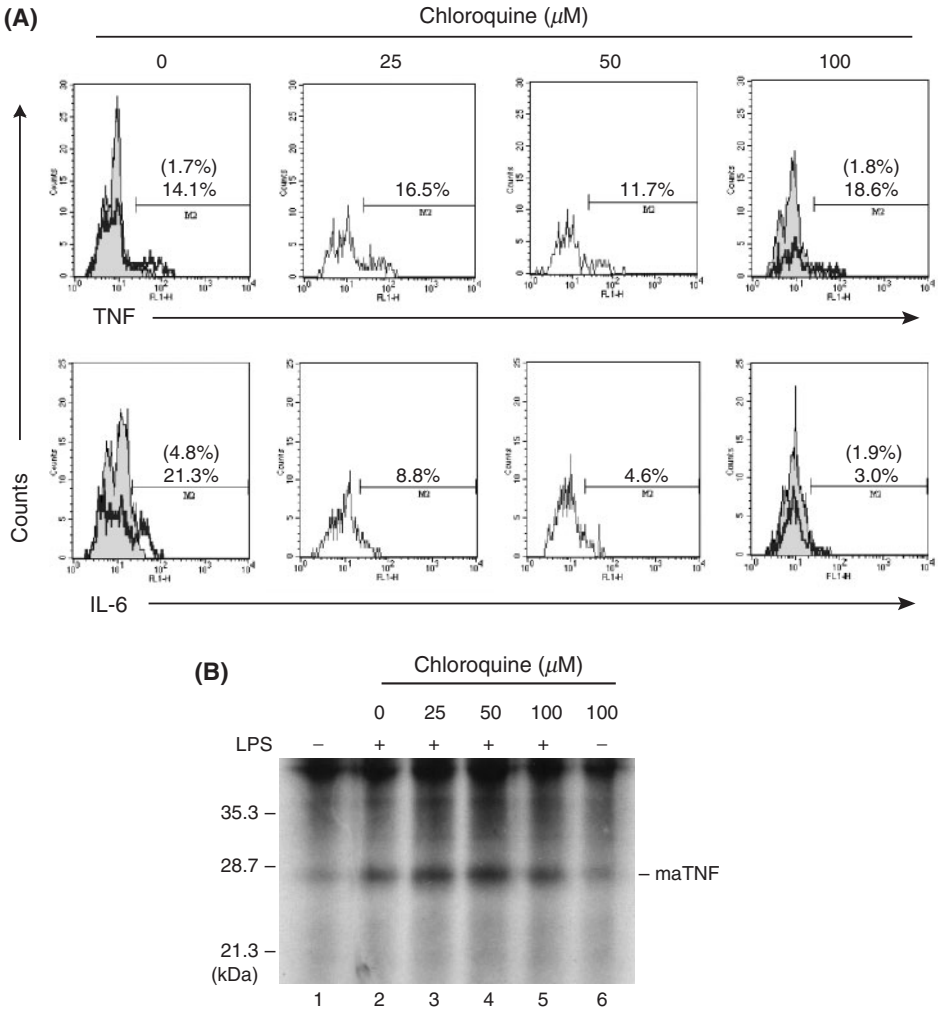

FIG. 2. Chloroquine does not block synthesis of 26-kDa TNF-α precursor. (A) Flow cytometry analysis of cell-associated TNF-α and IL-6. PBMCs were incubated in the absence (shaded) or presence of LPS (open) and various concentrations of chloroquine. After 4 h, cells were stained with anti-CD14-PE antibody, permeabilized, and stained with FITC-conjugated anti-TNF-α or anti-IL-6 antibody, and analysed by flow cytometry. The percentage of cells positively stained with anti-TNF-α or anti-IL-6 antibody (M2 region) among $CD14^+$ cells is presented, and the percentages of unstimulated cells are given in parenthesis. (B) PBMCs were incubated in the absence (lanes 1 and 6) or presence (lanes 2–5) of LPS and indicated concentrations of chloroquine for 2 h. After being labelled with [^{35}S]methionine/cysteine mix, cells were lysed and membrane-associated 26-kDa TNF-α precursor (maTNF) in the lysate were immunoprecipitated with anti-TNF-α antibody. Samples were analysed by SDS-PAGE and visualized by fluorography. The positions of molecular weight markers and membrane-associated TNF-α precursor (maTNF) are indicated. The data represent two independent experiments.

medium by ELISA and their mRNA level by northern blot analysis after LPS stimulation of cells. As shown in Fig. 3A, treatment of PBMCs and monocytes/macrophages with chloroquine decreased IL-1β and IL-6 release. As reported previously [28], a large amount of IL-1β (probably in the form of proIL-1β) was retained in the cell, and the level of cell-associated IL-1β was also decreased by chloroquine. In contrast to its effect on TNF-α mRNA, chloroquine treatment decreased IL-1β and IL-6 mRNA levels in a concentration range that inhibits protein synthesis (Fig. 3B). Similar inhibitory effects of chloroquine on IL-1β and IL-6 synthesis and their mRNA levels were observed in experiments performed with differentiated U937 and THP-1 cells (data not shown).

Chloroquine reduces stability of IL-1β and IL-6 mRNA

We performed nuclear run-on analysis to determine whether chloroquine modulates transcriptional activity of the TNF-α, IL-1β and IL-6 genes in LPS-stimulated monocytes/macrophages (Fig. 4A, B). Transcriptional activity of the TNF-α and IL-1β genes was increased about 50 and 500%, respectively, by LPS, whereas that of the IL-6 gene was elevated even in non-stimulated cells and was not further increased by LPS. Addition of chloroquine did not significantly change the transcriptional activity of these cytokine genes in the LPS-stimulated or non-stimulated cells. We then determined the stability of TNF-α, IL-1β and IL-6 mRNA in chloroquine-treated cells in order to investigate post-transcriptional regulation of their expression by chloroquine. In THP-1 cells stimulated with LPS for 4 h, the kinetics of the decay of TNF-α, IL-1β and IL-6 mRNA was analysed by RT-PCR after treating the cells with actinomycin D to terminate *de novo* transcription (Fig. 4C). TNF-α mRNA was degraded with a half-life of about 8 h, and it was not significantly altered by chloroquine. This is in contrast to IL-6 mRNA, whose half-life of about 8 h in control cells was significantly decreased to about 4 h in chloroquine-treated cells. Degradation of IL-1β mRNA was also

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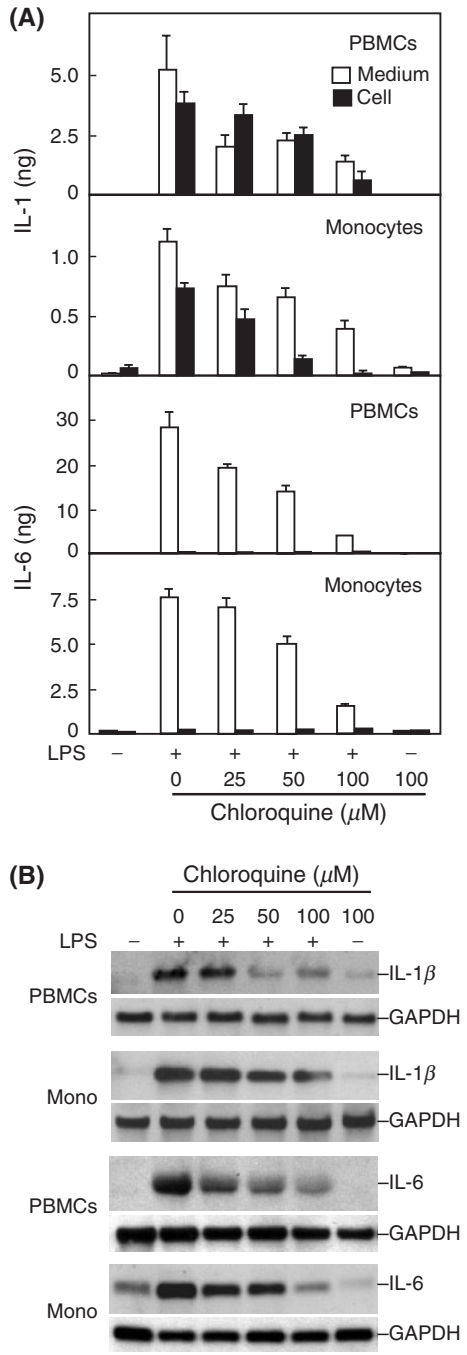

FIG. 3. Chloroquine decreases IL-1β and IL-6 synthesis and their mRNA levels. PBMCs, and monocytes/macrophages were incubated as described in Fig. 1. IL-1β and IL-6 in the culture medium and cell lysate were measured by ELISA (A) and mRNAs were measured by northern blot analysis (B). The data represent three independent experiments.

accelerated in cells incubated with chloroquine, although the change in degradation rate caused by chloroquine was less remarkable than that of IL-6 mRNA.

Inhibitory effect of chloroquine on IL-1β and IL-6 synthesis occurs partially through its weak-base property

Chloroquine, as a weak-base amine, is known to inhibit endolysosomal functions by neutralizing the acidity of these organelles [29]. We examined whether this lysosomotropic property of chloroquine is associated with its inhibitory action in TNF-α, IL-1β and IL-6 synthesis by testing the effect of other weak-base amines and the lysosomal protease inhibitor leupeptin on the synthesis of these cytokines in LPS-stimulated monocytes/macrophages (Fig. 5). Hydroxychloroquine, a hydrophilic congener of chloroquine, showed an inhibitory activity that was more potent than or comparable to that of chloroquine. On the other hand, methylamine and ammonium chloride had no effect on the synthesis of TNF-α, while they partially blocked IL-1β and IL-6 synthesis, methylamine being the more effective of the two. The lysosomal protease inhibitor leupeptin, which has no of pH-elevating effect, did not inhibit synthesis of these cytokines. We observed a similar inhibition of cytokine synthesis by the lysosomal inhibitors in experiments performed with THP-1 cells (data not shown).

Discussion

In this study, we showed that inhibition of TNF-α synthesis by chloroquine occurs at a post-translational step rather than a transcriptional step. In human primary PBMCs, and monocytes/macrophages and monocytic THP-1 and U-937 cells stimulated with LPS, chloroquine treatment commonly reduced the amount of TNF-α released into the culture medium but did not alter the level of TNF-α mRNA and the transcriptional activity of the TNF-α gene when determined by northern blotting and nuclear run-on analysis, respectively (Figs 1 and 4). Moreover, analysis of cell-associated TNF-α by ELISA and flow cytometry and immunoprecipitation analysis of metabolically labelled TNF-α indicated that the level or synthesis of cell-associated 26-kDa TNF-α precursor is not decreased by chloroquine (Fig. 1 and 2). These results agreed well with our previous study performed in mouse macrophage RAW 264.7 cells [18], and suggest that the inhibitory effect, which appears after synthesis of the 26-kDa TNF-α precursor, is common between human and mouse monocytes/macrophages. However, our results contradict those of other studies, which showed a chloroquine-induced decrease in TNF-α mRNA in human PBMCs [19, 20] and suppression of TNF-α promoter-driven reporter expression in THP-1 cells [21]. In addition, chloroquine has been shown to block LPS-induced activation of mitogen-activated protein kinase ERK in PBMCs, suggesting that inhibition of ERK-mediated LPS signalling is responsible for the chloroquine-induced decrease in transcriptional activity of the TNF-α gene [21]. In our experiment with THP-1 cells, however, we did not observe any change in the activation of ERK1/2 or MEK1/2, an upstream kinase of ERK, by chloroquine (unpublished result, C.H.C. and D.M.J.). This discrepancy between our result and other studies does not seem to be caused by a difference in cell types, because PBMCs and THP-1 cells were commonly used. Although the cause of this discrepancy is not clear, differences in cell source and culture conditions might be responsible for the different results. Collectively, our result showed that TNF-α mRNA is not decreased by chloroquine either in primary cells or in monocytic transformed cells.

Our results showed that the level of membrane-associated 26-kDa TNF-α is not decreased by chloroquine (Fig. 2), and this membrane-associated TNF-α was shown to be active in that it is capable of lysing TNF-α-sensitive target cells [30, 31]. Excessive overexpression of membrane-associated TNF-α in mice by genetic manipulation or by administration of TNF-α-converting enzyme (TACE) inhibitor induced arthritis and exacerbation of inflammatory liver diseases [32–34]. However, mice manipulated to express membrane-associated TNF-α at a normally regulated level were shown to be fully protected from endotoxic shock and experimental autoimmune encephalomyelitis [35, 36]. This suboptimal activity of membrane-associated TNF-α in acute and chronic inflammatory diseases might reflect the relatively low level of

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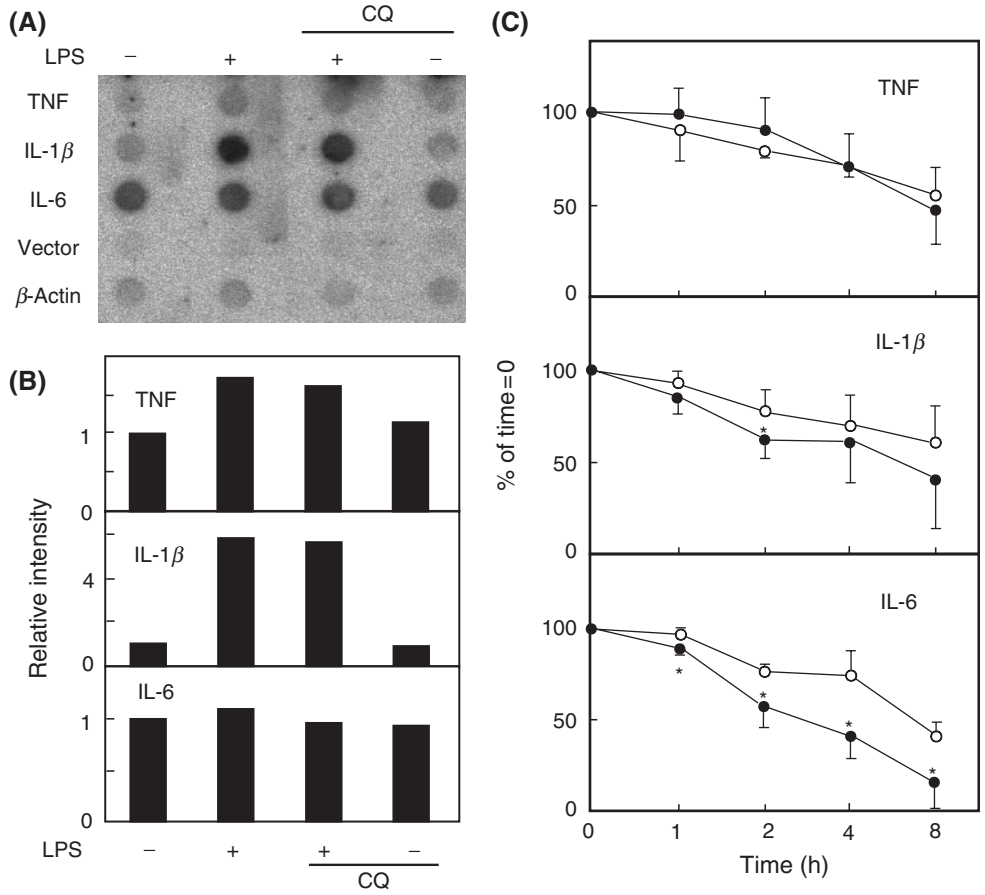

FIG. 4. Effect of chloroquine on transcriptional activity of TNF-α, IL-1β and IL-6 genes and their mRNA stability. (A) The transcription-competent nuclei were prepared from monocytes/macrophages stimulated with LPS (1 μg/ml) for 2 h. Nuclear run-on analysis was performed using a probe for TNF-α, IL-1β, IL-6, pGEM-T vector and β-actin spotted onto nylon membrane. (B) The radioactivity of each spot in A was quantitated by phosphor imager analysis and normalized with that of β-actin, and the transcriptional activity of each gene is presented relative to that of non-stimulated cells. The data represent three independent experiments. (C) THP-1 cells stimulated with LPS (10 μg/ml) for 4 h in the presence (closed circles) or absence (open circles) of 100 μM chloroquine were treated with actinomycin D (5 μg/ml) together with LPS and chloroquine for the indicated periods. Total RNA was isolated and mRNA levels were determined by RT-PCR using specific primer sets, and are shown after normalization with respect to those of β-actin. The data in A and B represent three independent experiments and those in C represent two independent experiments.

membrane-associated TNF-α compared with that of secreted 17-kDa TNF-α [5]. In our measurement, the level of cell-associated TNF-α was only about 1% of that of secreted TNF-α in cultures of PBMCs and monocytes/macrophages stimulated with LPS for 6 h (Fig. 1A and data not shown). Chloroquine induced an increase in cell-associated TNF-α only slightly (Fig. 2), and our previous study showed that the level of cell surface TNF-α in chloroquine-treated macrophages is similar to that in untreated control cells [18]. These results suggest that the membrane-associated TNF-α remaining in chloroquine-treated cells exhibits at most minor proinflammatory functions.

In contrast to the results obtained with TNF-α, inhibition of IL-1β and IL-6 synthesis by chloroquine was accompanied by decreases in their mRNA levels. Suppression of IL-1β and IL-6 synthesis and their mRNA levels by chloroquine in the same concentration range suggests that the change in the mRNA level is indeed responsible for the decreased production of these cytokines (Fig. 3). Our result is in agreement with that of a previous report [19], and suggests that chloroquine modulates some step(s) in the synthesis and metabolism of IL-1β and IL-6 mRNA: transcription of IL-1β and IL-6 genes, the processing of primary transcripts in the nucleus, the transport of processed mRNA to the cytosol, and the degradation of mRNA. In our nuclear run-on analysis, transcriptional activities of the IL-1β and IL-6 genes in LPS-stimulated monocytes/macrophages were not significantly altered by chloroquine, suggesting that chloroquine does not affect the synthesis of primary transcripts of these cytokines. By contrast, the stability of IL-1β and IL-6 mRNA was shown to be decreased by chloroquine. Although it is possible that chloroquine also modulates other steps of mRNA metabolism, our result indicates that accelerated decay may be responsible, at least in part, for chloroquine-induced decreases in IL-1β and IL-6 mRNA levels.

In our nuclear run-on assays, the transcription rate of the IL-6 gene was elevated even in cells that were not stimulated with LPS (Fig. 4A, B). This increased transcription rate of the IL-6 gene in monocytes/macrophages was consistently observed in repeated experiments, and also in experiments done with U-937 and THP-1 cells that were induced or not induced to differentiate into macrophages by incubating with PMA (data not shown). In previous studies, nuclear run-on assays of IL-6 gene transcription in PBMCs and monocytes showed that the basal low rate of transcription increased upon LPS stimulation [37, 38]. This discrepancy between our results and those of other groups might indicate an unknown problem in our assay method. On the contrary, it is possible that the increase in IL-6 mRNA in

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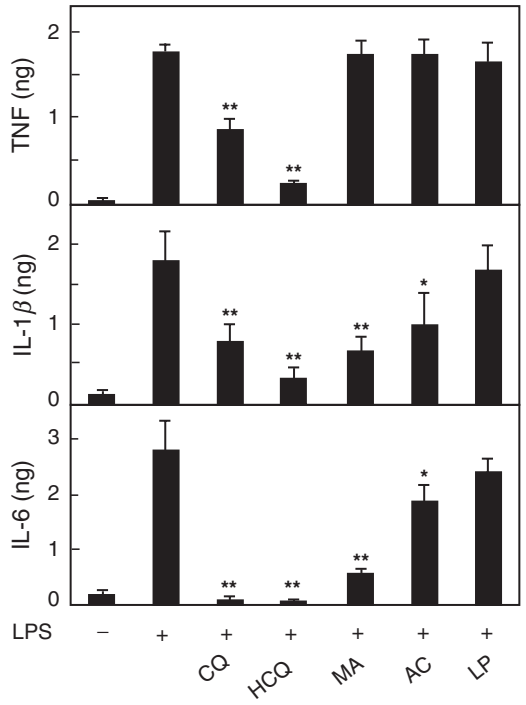

FIG. 5. Weak-base property of chloroquine is partially responsible for inhibition of IL-1β and IL-6 synthesis. Monocytes/macrophages were treated with chloroquine (CQ, 100 μM), hydroxychloroquine (HCQ, 100 μM), methylamine (MA, 10 mM), ammonium chloride (AC, 10 mM) or leupeptin (LP, 250 μM) for 2 h, and stimulated with LPS (1 μg/ml) for 6 h. Levels of TNF-α, IL-1β and IL-6 released into the culture medium were measured by ELISA. Statistical significances of differences were determined using Bonferroni's multiple comparison method and the results are expressed as mean $\pm$ S.D. ($n = 4$). $^*P < 0.05$; $^{**}P < 0.01$ compared with LPS-stimulated control cells. The data represent three independent experiments.

LPS-stimulated monocytes/macrophages depends largely on a post-transcriptional control mechanism in our experimental conditions.

Our experiment with lysosome/endosome inhibitors further supports our view that the action of chloroquine in the inhibition of TNF-α, IL-1β and IL-6 synthesis operates through different modes. Because of its weak-base property, chloroquine is known to neutralize cellular acidic compartments, including lysosomes and endosomes, and alter the targeting and intracellular trafficking of newly synthesized proteins [29, 39]. When we added other weak base amines (methylamine and ammonium chloride) to the culture medium of monocytes/macrophages and THP-1 cells instead of chloroquine, we did not observe any inhibition of TNF-α production by these agents. This result shows that the weak-base property of chloroquine is not involved in the inhibition of TNF-α synthesis, and confirms the results of previous studies performed with RAW 264.7 cells [18] and human PBMC [20]. On the contrary, our result showed that the production of IL-1β and IL-6 was decreased by methylamine and ammonium chloride, albeit to a lesser degree than by chloroquine and hydroxychloroquine. In a previous study, weak-base amines such as chloroquine and ammonium chloride were shown to interfere with the secretion of IL-1β through a pathway involving endolysosome-related vesicles in LPS-stimulated monocytes [40]. Thus, it is possible that the inhibition of IL-1β production induced by weak-base amines occurs by blocking secretion of proIL-1β rather than by decreasing IL-1β mRNA. However, our measurement of IL-1β and IL-6 mRNA levels showed that methylamine and ammonium chloride, like chloroquine, induce decreases in mRNA levels to a similar extent to the decrease in secreted IL-1β and IL-6 levels (data not shown). These results suggest that chloroquine and other weak-base amines inhibit IL-1β and IL-6 synthesis largely by decreasing their mRNA levels and that a certain pH-sensitive step is involved in the processing and maintenance of IL-1β and IL-6 mRNA.

In this study, the inhibitory effects of chloroquine and hydroxychloroquine were tested in cells incubated with these agents for 2 h, whereas *in vivo* plateau concentrations of the drugs—and thus their therapeutic effect—are attained after 1–2 months of treatment [41]. Concentrations of the drugs used in our *in vitro* study (25–100 μM) seem to be much higher than would be expected *in vivo*, because the plasma concentration was reported to be 0.6–0.9 μM in patients treated with these drugs [41, 42]. However, the levels of chloroquine in the liver, spleen and leucocytes of the patients are much higher than those in other organs, and are between 100 and 300 μM [42]. A previous study showed that the intracellular level of hydroxychloroquine in mononuclear cells from patients receiving a standard dose of 400 mg daily for at least 3 months was similar to that in mononuclear cells incubated *in vitro* with 100 μM chloroquine or hydroxychloroquine for 1 h [43]. These results strongly suggest that therapeutic doses of chloroquine and hydroxychloroquine indeed play roles in the suppression of TNF-α, IL-1β and IL-6 production in patients treated with the drugs.

In summary, our result shows that chloroquine inhibits TNF-α, IL-1β and IL-6 synthesis by different modes in LPS-stimulated human PBMCs and monocytes/macrophages, and monocytic U937 and THP-1 cells. The inhibitory effect of chloroquine on TNF-α synthesis appeared at a step in the processing of pro-TNF-α and the secretion of mature protein, whereas its effect on IL-1β and IL-6 synthesis was shown to be associated with reduced mRNA stability and mRNA level. Chloroquine and hydroxychloroquine have been used successfully in the treatment of RA [13, 14] and other inflammatory diseases, such as systemic lupus erythematosus [44]. Understanding the inhibitory mode of chloroquine in the production of proinflammatory cytokines from immune cells will benefit the development of novel strategies to treat these diseases.

Rheumatology	Key message
	• The anti-rheumatic drug chloroquine inhibits TNF-α production from monocytes/macrophages by blocking conversion of cell-associated TNF-α precursor to mature protein, whereas it inhibits IL-1β and IL-6 synthesis by decreasing the stability of their mRNAs.

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