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Hydroxychloroquine Sulphate Inhibits *In Vitro* Apoptosis of Circulating Lymphocytes in Patients with Systemic Lupus Erythematosus

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SUMMARY **The serological hallmark of systemic lupus erythematosus (SLE) is the presence of antibodies against double-stranded DNA. However, several studies have suggested that it is not DNA itself, but nucleosomes that are the immunogenic particles involved both in the induction of anti-DNA antibodies, and in the pathophysiology of SLE. Meanwhile, it has been demonstrated that there is an accelerated *in vitro* apoptosis of lymphocytes from patients with SLE. Therefore, one can postulate that the process of apoptosis may provide a source of nuclear antigens to drive the autoantibody response seen in SLE. Our study has demonstrated that hydroxychloroquine exhibits an anti-apoptotic action and this anti-apoptotic effect is dependent on monocyte coexistence. We used both morphology assessment and fluorescent antibody cell sorter (FACS) analysis to measure the apoptotic percentage of lymphocytes from 25 SLE patients in medium alone (control) or with the addition of different concentrations of hydroxychloroquine. Our results have shown that there is a significant decrease in the percentage of apoptosis at the therapeutic concentration (10^{-6} M) as compared with the control ($p < 0.05$). It has been reported that the antirheumatic properties of hydroxychloroquine result from its interference with antigen processing in macrophages and other antigen-presenting cells. We propose that this results in decreased stimulation of autoreactive lymphocytes reactive with self-peptides, and consequently diminution of activation-induced cell death (apoptosis) of mature peripheral lymphocytes.**

Systemic lupus erythematosus (SLE) is characterized by the production of autoantibodies to multiple nuclear antigens. The cardinal serologic feature of SLE is the presence of antibodies to double-stranded DNA. It has been proposed that these anti-DNA antibodies are involved in the development of lesions in SLE. In recent years it has become evident that nucleosomes (DNA complexed to histone), and not free DNA, participate both in the pathophysiology of SLE and in the induction of anti-DNA antibodies. The recent identification of the genetic lesions in certain murine models of lupus-like diseases (lpr and gld mice),[1-4] and perhaps in SLE patients as well, suggests that the process of apoptosis is aberrant. Emlen *et al.*[5] have demonstrated that apoptosis of lymphocytes from SLE patients is accelerated *in vitro* and that, because of this accelerated apoptosis, increased amounts of nucleosomes are released into the extracellular milieu; both of these findings significantly correlate with SLE disease activity.[5] These findings raise the possibility that increased apoptosis might lead to the release of large amounts of potentially immunostimulatory intracellular antigens, in particular nucleosomes, thereby providing sufficient antigen to drive an autoimmune response and to form immune complexes.

Most clinicians would agree that antimalarial agents reduce flares

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and are corticosteroid sparing. In fact, a recent double-blind placebo-controlled study has clearly shown that patients with quiescent SLE who took hydroxychloroquine were less likely to have a clinical flare-up if they were maintained on the drug.[6] As the antimalarial compounds are weak bases that lack protons at the neutral pH of serum, they can diffuse into acidic vacuoles, where they become protonated. The more polar molecules are unable to diffuse out of the vacuoles, the more they will elevate the pH within the vacuoles. The elevated pH, in turn, alters the molecular assembly of α-β-peptide complexes. This may interfere with antigenic processing in antigen-presenting cells (APC) and lead to a reduced activation of autoreactive lymphocytes, resulting in a down-regulation of autoimmune responses.[7] Based on the theoretical mechanism of action of hydroxychloroquine, it seems reasonable to postu late that hydroxychloroquine exerts its effects through the modulation of apoptosis.

This study was carried out to assess the proportion of apoptotic lymphocytes from SLE patients in medium alone (control), or with the addition of different concentrations of hydroxychloroquine ranging from 10^{-7} to 10^{-5}M. The present study shows that *in vitro* addition of hydroxychloroquine at the therapeutic concentration (10^{-6} M) to lymphocytes from SLE patients decreases the proportion of apoptotic cells.[8] Meanwhile, this anti-apoptotic effect is dependent on monocyte coexistence.

MATERIALS AND METHODS

Selection of patients

Twenty-five females aged 27.3 ± 7.6 years who fulfilled at least 4 criteria of the American College of Rheumatology (ACR) for the diagnosis of SLE were enrolled in this study.[9] All of these patients were regularly followed up at the rheumatological clinic of the National Taiwan University Hospital. The disease duration ranged from 1 year to more than 5 years. Patients enrolled had not taken hydroxycholoroquine, steroids or cytotoxic agents for at least 3 months, owing to inactivity of the disease.

Isolation and culture of peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells were purified from 15 ml heparinized peripheral blood by Ficoll gradient according to Boyum.[10] The cells were then washed in an RPMI 1640 medium and cultured at 37°C in 5% CO_2 in 24-well microtiter plates. The cells were re-suspended in RPMI 1640/ 10% fetal calf serum (FCS) supplemented with 2 mM glutamine, 100 units penicillin, 100 μg streptomycin/ml, and then adjusted to 10^6 cells/well in medium alone (control), or in medium plus hydroxychloroquine (10^{-6} or 10^{-7} M) (Sanofi-Winthrop, NY, USA).

Cell viability assay

Prior to initiating the assessment of apoptosis, the number of surviving cells was determined visually by counting adhering cells that excluded trypan blue under the microscope.

Measurement of apoptosis

We have used two different methods, *i.e.* morphology and flow cytometry analysis, to assess the apoptosis of lymphocytes from SLE patients. Aliquots of cells were removed at 24 and 48 hours after initiating of culture.

Morphology assay

Apoptosis was assessed by the appearance of nuclear condensation or fragmentation at different times after plating in medium alone (control) or in medium with different concentrations of hydroxychloroquine. Immediately after plating (time 0), at 24 and 48 hours, the samples were removed. Lymphocytes with morphologically apoptotic cells were stained with acridine orange (AO) by adding 1 μl of AO solution (100 μg/ml) to a 25 μl cell suspension.[11] After mixing thoroughly, the cells were fixed by adding 20 μl of 1% paraformaldehyde, and were then analyzed by fluorescence microscopy. We calculated the percentage of apoptotic cells as follows: % apoptotic cells = total no. of cells with apoptotic nuclei/ total no. of cells counted x 100. At least 200 cells were counted per sample point by two observers. Interobserver agreement was good, because the difference in the results between the two observes was always less than 5%.

Quantification of apoptosis by flow cytometry (FACS analysis)

Cells were lysed and stained with propidium iodide (PI) according to Nicoletti *et al.*[12] and analyzed using specific FACScan research software (Becton Dickinson). In brief, because apoptotic cells either contain reduced levels of DNA, or contain fluorochrome-inaccessible DNA (caused by chromatin condensation), the nuclei of apoptotic cells take up less PI than

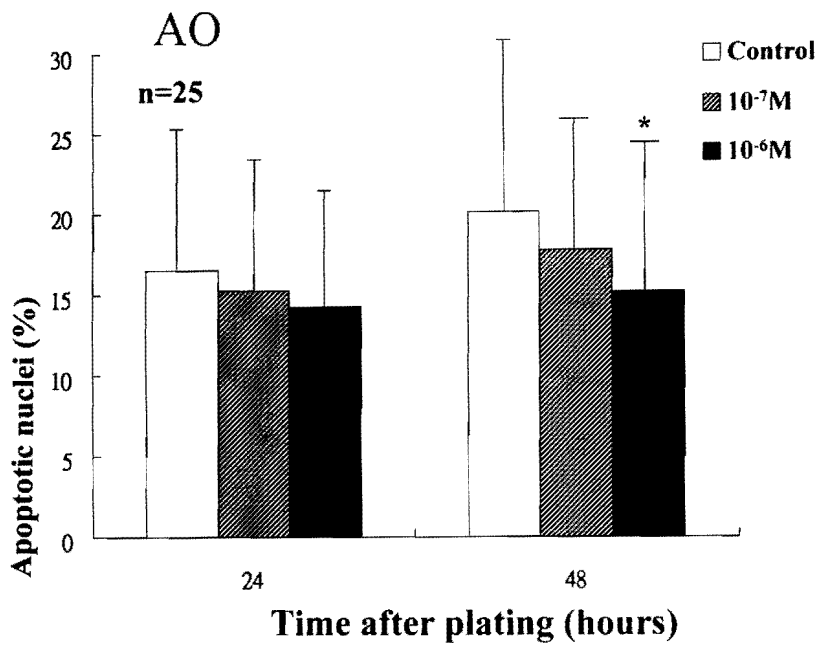

Fig. 1 Effect of different concentrations of hydroxychloroquine on nuclear fragmentation. The morphological characteristics of apoptosis in medium alone, in the presence of 10^{-7} M or 10^{-6} M of drug was examined at 24 hours and 48 hours. Only lymphocytes treated with 10^{-6} M of drug showed a significantly lower percentage of apoptotic cells compared with those of the control at 48 hours ($p < 0.05$). AO: acridine orange.

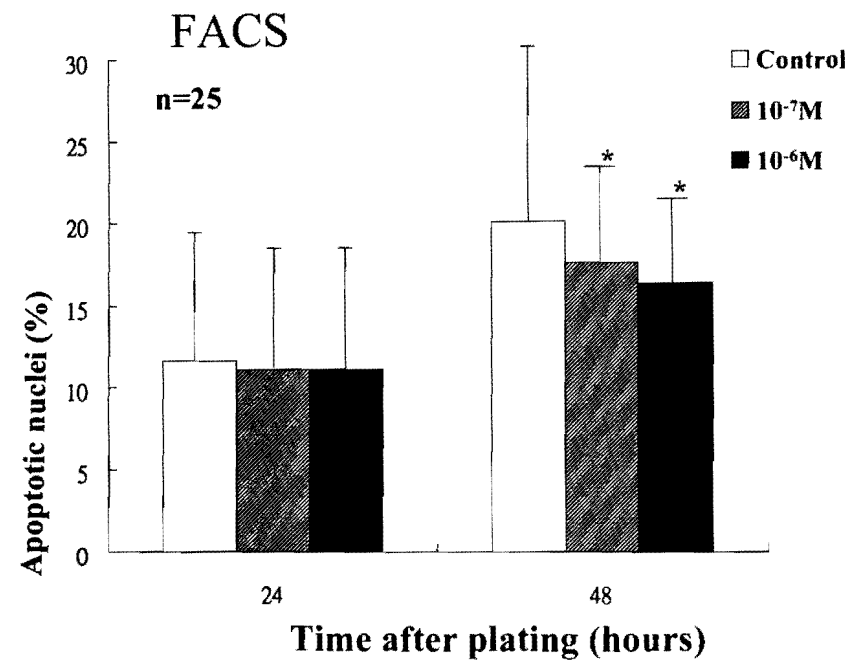

Fig. 2 Effect of different concentrations of hydroxychloroquine on nuclear fragmentation analyzed by FACScan. Lymphocytes from 10^{-7} or 10^{-6} M of drug showed a significantly lower percentage of apoptotic cells than did lymphocytes from the control at 48 hours ($p < 0.05$).

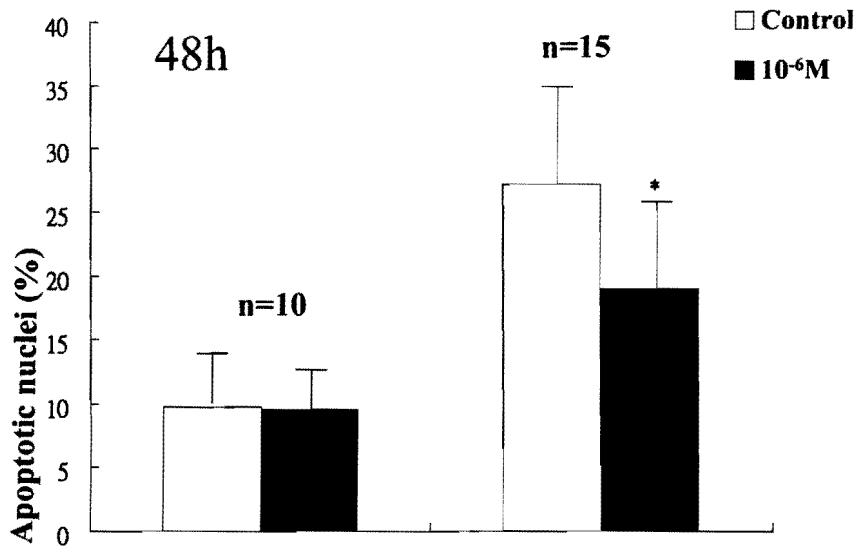

Fig. 3 Effect of the severity of apoptosis on the anti-apoptotic action of hydroxychloroquine. Patients were divided into two groups according to the severity of apoptosis observed in medium alone at 48 hours: One group had the percentage of apoptosis above 15% (n = 15), the other group with the percentage below that value (n = 10). Only the former showed a significant decrease in the percentage of apoptosis in the presence of 10^{-6} M of drug ($p < 0.05$).

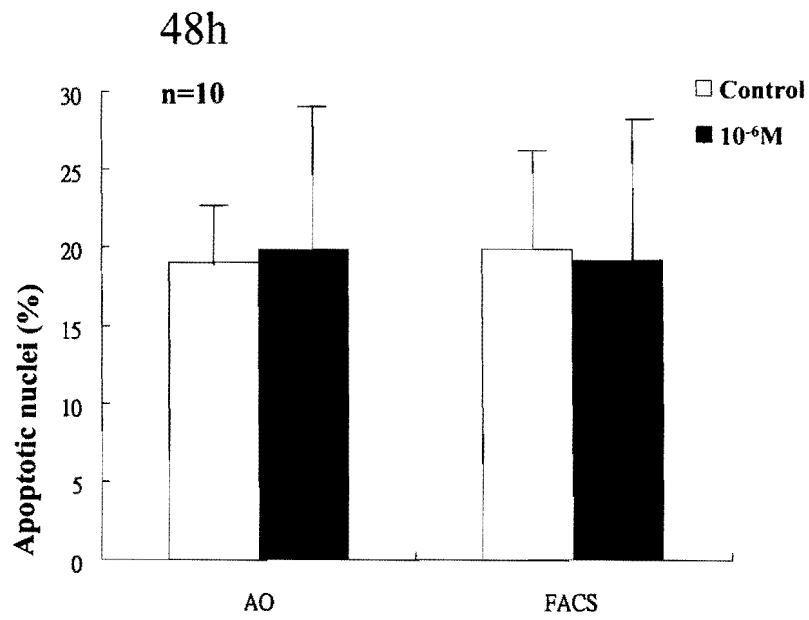

Fig. 4 Effect of the severity of apoptosis on the anti-apoptotic action of hydroxychloroquine (cont.). After removal of monocytes, lymphocytes exposed to 10^{-6} M of drug failed to show a significantly lower percentage of apoptotic cells than did lymphocytes from the control group.

the nuclei of normal cells, resulting in apoptotic nuclei appearing as a hypodiploid DNA peak, which can be distinguished from the narrow peak of cells with normal (diploid) DNA content.

Statistical analysis

The mean ± standard deviation was used in the expression of percentages of apoptotic cells in the study. Mann-Whitney test was used to compare apoptotic percentages between different groups of patients. Values of $p < 0.05$ were chosen for rejection of the null hypothesis.

RESULTS

The viability testing showed that 10^{-5} M hydroxychloroquine resulted in 18% cytotoxity at the end of the incubation period; lower concentrations of the drug did not affect cell viability.

Measurement of apoptosis by AO staining

The percentage of apoptotic lymphocytes was less than 5% at time 0. At 24 hours, 16.5 ± 8.6% of lymphocytes from the control were apoptotic and at 48 hours, 20.1 ± 11%. In the presence of 10^{-6} M hydroxychloroquine, 14.2 ±7.5% of lymphocytes were apoptotic at 24 hours ($p > 0.05$) and 15.1 ± 5.2% at 48 hours ($p < 0.05$) (Fig. 1). These data indicate that there was a significant decrease in the percentage of apoptosis in medium with 10^{-6} M hydroxychloroquine as compared with that of the control. In the presence of 10^{-7} M hydroxychloroquine, the percentage of apoptotic cells was 15.2 ± 7.8% at 24 hours ($p > 0.05$) and 17.7 ± 8.9% at 48 hours ($p > 0.05$).

Measurement of apoptosis by FACScan

Flow cytometry analysis of PI-stained lymphocytes from parallel cultures was performed at 0, 24 and 48 hours. At 24 hours, there was no significant difference between the control and the hydroxychloroquine-treated cells. At 48 hours, 20.1 ± 11.6% of lymphocytes from the control were apoptotic, 17.6 ± 5.7% with 10^{-7} M hydroxychloroquine ($p < 0.05$), and 16.3 ± 5% with 10^{-6} M hydroxychloroquine ($p < 0.05$) (Fig. 2). At a concentration of 10^{-7} M, FACScan analysis, in contrast to AO staining, revealed a significant decrease in the percentage of apoptosis.

Furthermore, according to the severity of apoptosis observed in the medium alone (control) at 48 hours, we divided these patients into two groups: in one group, the percentage of apoptosis was above 15% (n = 15); in the other group, the percentage was below that value (n = 10). By virtue of morphology assays, we found that the former group showed a significant decrease in the percentages of apoptosis in the presence of 10^{-6} M hydroxychloroquine, whereas the latter failed to demonstrate the same result (Fig. 3). A similar result was obtained from FACScan analysis (data not shown). These results might imply that the degree of reduction in the percentage of apoptosis tended to be significant if the proportion of apoptotic lymphocytes from the control was elevated abnormally (e.g. > 15%).

DISCUSSION

FACS analysis, but not AO staining, demonstrated a significant reduction in the percentage of apoptosis in the presence of 10^{-7} M of hydroxychloroquine at 48 hours. The discrepancies observed between FACScan and AO staining analysis may be explained as follows: in a cycling population, cells undergoing apoptosis during S or G_2/M phases of the cell cycle also emit lower levels of DNA fluorescence than do their viable counterparts. Such cells merge with the viable G_1-and S-phase populations and consequently remain undetected.[13] These effects result in an underestimation of the true size of the apoptotic population when the percentage of cells in the subdiploid peak is compared with the percentage of apoptotic cells scored by microscopic counting of AO-stained cells. Such FACS analysis should be used only after diagnosis of apoptosis by a complementary technique such as AO staining.[14] As a result, the outcome of the present study implied that the anti-apoptotic effect of hydroxychloroquine was only seen when the dose of the drug used throughout the culture was 10^{-6} M.

What are the potential mechanisms by which hydroxychloroquine can exert this anti-apoptotic effect? *In vitro* studies have suggested a wide range of activities for this compound. These include the ability to inhibit some phospholipids, act as antioxidant, intercalate into DNA, and interfere with intracellular pH in cytoplasmic vacuoles. However, the most likely mechanism of antirheumatic activity is the elevation of intracytoplasmic pH. This may interfere with antigen processing in APC and result in downregulation of autoimmune responses. Based on the proposed mechanism of antirheumatic activity of antimalarials, we attempt to explain the therapeutic effect of hyroxychloroquine by this anti-apoptotic activity.

Recent studies using T-cell receptor (TCR)-transgenic mice deficient in ATP-dependent peptide transporter (TAP-1) expression have suggested a differential-avidity model of thymic selection. The rule has emerged that low-avidity interaction between the TCR and the major histocompatibility complex (MHC)/peptide leads to positive selection, whereas high avidity drives negative selection.[15,16] However, the affinity of an epitope for its MHC restriction element may likewise prove critical: several recent studies have described how epitopes displaying only negligible affinity for MHC class II might occasionally become immunodominant in preference to other epitopes known to display for higher affinity.[17] All such low-affinity epitopes are generated from self-proteins rather than being derived from foreign antigens.

In the thymus, thymocytes specific for self-peptides with high affinity for their MHC restriction element are clonally deleted by negative selection. While thymocytes with no affinity for self-peptide bound to MHC fail to be rescued from apoptosis by positive selection. By contrast, thymocytes specific for self-peptides with low affinity for MHC determinants may escape negative selection and populate the periphery. As a result, clonal deletion of T cells specific for high-affinity self-proteins in the thymus promotes low-affinity epitopes of apparent immunodominance in the periphery.[18]

We postulate that, in the periphery, certain T cells, in particular self-reactive T cells, responding to environmental antigens cross-react with low-affinity self-antigens (nucleosomes?). Chronic stimulation of these T cells by self-antigen leads to activation-induced cell death. On the other hand, owing to low-affinity self-antigen, autoreactive B cells that are activated by CD40 ligand, delivered by autoreactive T cells in the absence of an effective signal through their surface immunoglobulin receptor, can be effectively eliminated probably by Fas-mediated apoptosis if they happen to be activated by autoreactive T cells. Because hydroxychloroquine elevates the intracytoplasmic pH, the dissociation of α-li and β-li is inhibited and, in turn, only peptides with high affinity for self-MHC restriction elements can replace li and form a complex. As a result, the density of self-peptides with low affinity for MHC restriction element on the APC surface will decrease and the activation of autoreactive lymphocytes will also be down-regulated. Consequently, apoptosis of T and B cells will be down-regulated.

To verify that the anti-apoptotic effect of hydroxychloroquine is dependent on macrophages for their interference with antigen processing, an additional 10 female patients were recruited. After peripheral blood mononuclear cells had been isolated by the Ficoll-Hypaque technique, these cells were re-suspended in RPMI/5% autologous serum at 37°C for 1 hour to allow the monocytes to adhere. Nonadherent cells were removed, re-suspended, and used to measure apoptosis. There were more than 95% lymphocytes in the preparation by the FACScan analysis. However, under a condition of depletion of monocytes, there was no demonstrable decrease in the percentage of apoptotic lymphocytes in the presence of 10^{-6} M of hydroxychloroquine at 48 hours as measured by both methods ($p > 0.05$) (Fig. 4).

Findings of a recent study suggested that hydroxychloroquine induced apoptosis in peripheral blood lymphocytes.[19] Our results were not in conflict with the previous study. The apoptosis was assayed in isolated mononuclear cells following incubation for 1 hour to allow monocytes to adhere. Nonadherent cells were used to assess apoptosis, *i.e.* being assessed underdetectable in a condition of depletion of monocytes. Under a condition of depletion of monocytes, we were unable to demonstrate the same result because of our small study population.

In conclusion, we showed that, in the presence of a therapeutic serum concentration of hydroxychloroquine, the proportion of apoptotic lymphocytes from SLE patients decreased significantly. One possible mechanism is that hydroxychloroquine diminishes the formation of low-affinity self-peptide-MHC complexes required to activate autoreactive lymphocytes, and leads to the down regulation of the immune response against autoantigenic peptides. We postulate that this, in turn, results in decreased apoptosis of lymphocytes in SLE patients. Further studies to precisely identify the site of action are needed. Our observations may provide new insights toward understanding the mechanism of action of hydroxychloroquine.

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