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Effect of hydroxychloroquine treatment on pro-inflammatory cytokines and disease activity in SLE patients: data from LUMINA (LXXV), a multiethnic US cohort

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Abstract

Objective—We sought to determine the effect of hydroxychloroquine therapy on the levels proinflammatory/prothrombotic markers and disease activity scores in patients with systemic lupus erythematosus (SLE) in a multiethnic, multi-center cohort (LUMINA).

Methods—Plasma/serum samples from SLE patients (n=35) were evaluated at baseline and after hydroxychloroquine treatment. Disease activity was assessed using SLAM-R scores. Interferon (IFN)-α2, interleukin (IL)-1β, IL-6, IL-8, inducible protein (IP)-10, monocyte chemotactic protein-1, tumor necrosis factor (TNF)-α and soluble CD40 ligand (sCD40L) levels were determined by a multiplex immunoassay. Anticardiolipin antibodies were evaluated using ELISA assays. Thirty-two frequency-matched plasma/serum samples from healthy donors were used as controls.

Results—Levels of IL-6, IP-10, sCD40L, IFN-α and TNF-α were significantly elevated in SLE patients versus controls. There was a positive but moderate correlation between SLAM-R scores at baseline and levels of IFN-α (p=0.0546). Hydroxychloroquine therapy resulted in a significant decrease in SLAM-R scores (p=0.0157), and the decrease in SLAM-R after hydroxychloroquine therapy strongly correlated with decreases in IFN-α (p=0.0087).

Conclusions—Hydroxychloroquine therapy resulted in significant clinical improvement in SLE patients, which strongly correlated with reductions in IFN-α levels. This indicates an important role for the inhibition of endogenous TLR activation in the action of hydroxychloroquine in SLE and provides additional evidence for the importance of type I interferons in the pathogenesis of SLE. This study underscores the use of hydroxychloroquine in the treatment of SLE.

Keywords

Lupus; hydroxychloroquine; biomarkers of inflammation; biomarkers of thrombosis

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Conflict of interest statement

None declared.

Introduction

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease which may affect multiple organ systems, resulting in protean clinical manifestations.[1] Abnormal biological activity of cytokines has been observed in these patients and several studies have suggested the association of proinflammatory cytokines with disease activity and specific clinical manifestations.[2] An interferon (IFN) gene expression signature has been detected in peripheral mononuclear blood cells (PMBCs) of some SLE patients and possibly serves as a marker for more severe disease involving the renal, hematopoietic and/or the central nervous systems.[3] There is also evidence that levels of IFN and IFN-inducible chemokines/cytokines such as macrophage inflammatory protein-1 (MIP-1), monocyte chemotactic protein-1 (MCP-1) and interferon-inducible protein-10 (IP-10) are correlated with disease activity as measured by the different disease activity indices, the erythrocyte sedimentation rate (ESR) and anti-dsDNA antibody titers.[4,5] Other proinflammatory cytokines that have been shown to be correlated with disease activity in SLE patients include tumor necrosis factor-α (TNF–α), soluble CD40 ligand (sCD40L) and interleukin-6 (IL-6).[6,7]

Antiphospholipid (aPL), including anticardiolipin (aCL) and anti-β_2 glycoprotein I (anti-β_2 GPI), antibodies are also seen in approximately 30–40% of patients with SLE and approximately 50% of those patients develop antiphospholipid syndrome (APS).[8] On the other hand, IL-1, IL-6 and IL-8 and TNF-α have been shown to be upregulated by aPL antibodies in vitro and in animal models.[9,10]

Antimalarial drugs, namely hydroxychloroquine (HCQ) in North America and Western Europe, remain the first line of treatment for SLE because they exert beneficial hematological and immunological effects which translate to efficacy in preventing flares, treating cutaneous and musculoskeletal lupus, retarding the onset of damage in general, and damage in the cutaneous and renal systems, preventing some cardiovascular and central nervous system complications and improving survival in SLE patients.[11–14] Although it is known that HCQ has several immunomodulatory, anti-hyperlipidemic and anti-thrombotic effects, the exact mechanisms by which this drug modifies disease expression and progression in SLE patients remain largely unknown.[15–17]

However, there are limited data highlighting the effect that HCQ may have on biomarkers of disease activity. As such we sought to determine the proinflammatory biomarkers profile in patients from the LUMINA (LUpus in MInorities, NAture versus nurture) cohort, a longitudinal study of outcome in SLE patients and its relationship with disease activity.

Methods

Patients

Patients for inclusion in the study were selected from the LUMINA cohort. LUMINA is a longitudinal study of outcome of multiethnic [Hispanic (Mexican/Central American and Puerto Rican), African American and Caucasian] SLE patients enrolled within five years of fulfillment of the American College of Rheumatology (ACR) criteria at participating institutions in Alabama, Texas (Houston and Galveston) and Puerto Rico.[18,19] Patients had clinical and laboratory evaluations which included blood samples being drawn and disease activity assessments using the Systemic Lupus Activity Measure-Revised (SLAM-R) performed at six-month intervals for the first year of enrollment and annually thereafter. Patients placed on HCQ therapy during follow-up were included in this study if serum or plasma samples taken before and after the commencement of therapy, at least six months apart and stored at −20°C, were available for testing. Exclusion criteria included concurrent use of immunosuppressive drugs such as azathioprine, cyclophosphamide or prednisone at

doses greater than 10mg/day, and statins at baseline and at the follow-up visit. Serum samples taken from 32 frequency-matched controls with no evidence of autoimmune or inflammatory disease (85% females, age range 18–65 years) were also tested to serve as a comparison with baseline results in SLE patients.

The LUMINA study had been conducted following the declaration of Helsinki guidelines for inclusion of humans in research. All subjects had provided informed consent.

Antiphospholipid testing

aCL antibodies, IgG, IgM and IgA isotypes, were measured by an in-house enzyme-linked immunosorbent assay (ELISA) method as previously described.[20]

Cytokine testing

The serum or plasma levels of the cytokines IFN-α2, IL-1β, IL-6, IL-8, IP-10, MCP-1, TNFα andsCD40L were determined by the MILLIPLEXMAP human cytokine/chemokine panel assay (Millipore, Billerica, MA, USA) which utilizes Luminex xMAP technology. Briefly, *25μl* of patient serum or plasma was incubated with color-coded bead sets, each set having a distinct internal fluorescent dye and a distinct coat of capture antibodies specific for one of the analytes being tested. A biotinylated detection antibody was then introduced followed by incubation with streptavidin-phycoerythrin, which acted as the reporter molecule on the surface of each microsphere. Distinct lasers were used to excite the internal dyes marking each microsphere set and the dye in the reporter molecule followed by high speed digital signal processing to quantify the reporter signals from each bead set.

Statistical analyses

The Kruskal–Wallis test was used to compare cytokine levels in SLE patients with those in controls. A signed rank test was used to calculate the effect of drug treatment on cytokine and disease activity levels. Spearman correlation was used to compare changes in levels of biomarkers with changes in SLAM-R. All p values less than 0.05 were considered to be significant.

Results

Patient demographics and characteristics

After application of selection criteria 35 patients were included in the HCQ analyses group [89% were females and their mean (range) age was 33.9 (16–63) years]. Patients of African descent accounted for the majority of patients (23/35, 66%); other patients were Hispanic from Mexico or Central America (5/35, 15%) or from Puerto Rico (3/35, 8%), and Caucasian (4/35, 11%). Clinical characteristics of the subjects are depicted in Table 1.

Proinflammatory cytokines in SLE patients and controls

The levels of proinflammatory cytokines were strikingly different in SLE patients at baseline compared with controls. Median levels of IL-6 (9.84 *vs.* 0.00), IP-10 (426.15 vs. 100.84), sCD40L (1737.41 *vs.* 16.35), IFN-α (211.50 *vs.* 0.00) and TNFα (7.19 *vs.* 0.00) were significantly elevated in SLE patients at baseline versus controls (p<0.0001–0.0002).

There was a positive but modest correlation between SLAM-R scores and the levels of IFN-α (Spearman correlation coefficient 0.314, p=0.0546). No other biomarkers correlated with SLAM-R at baseline.

Effect of HCQ therapy

Table 2 depicts the changes of biomarker levels in SLE patients treated with HCQ. HCQ therapy produced a significant decrease in median SLAM-R scores (p=0.0157). Twenty-two patients (62.86%) had decreased SLAM-R scores in response to HCQ therapy, 10 patients (28.57%) had increased scores and three patients (8.57%) had no change in scores. The median levels of sCD40L, IL-6, IFN-α, IL-8 and TNF-α also decreased with HCQ treatment by 59.3%, 45.8%, 33.5%, 26.5% and 17.0%, respectively; however, these changes were not statistically significant. Median levels of aCL, IL-1β and IP-10 all either remained the same or increased following HCQ therapy. Interestingly there was a strong positive correlation between the decreases observed in IFN-α and SLAM-R after HCQ therapy (Spearman correlation coefficient 0.614, p=0.0087). There were no significant correlations between changes observed in the other biomarkers and SLAM-R scores.

Discussion

We provide evidence confirming the presence of elevated pro-inflammatory cytokines in SLE patients and a positive correlation between elevated IFN-α levels and disease activity. We also demonstrate that HCQ results in decreased disease activity levels in SLE patients as measured by SLAM-R scores. In this small cohort of SLE patients, although most cytokines and pro-inflammatory markers were lowered after HCQ therapy, these reductions were not statistically significant. However, we show for the first time that reduction in disease activity scores in HCQ-treated patients is positively correlated with reductions in IFN-α.

HCQ has been used for many years to treat patients with SLE since it is relatively inexpensive and well tolerated and there is much evidence to suggest that in addition to its usefulness in treating mucocutaneous, articular and constitutional manifestations in these patients it reduces serum cholesterol levels, disease flares and thrombotic complications.[11–14] Furthermore, previous studies in the LUMINA cohort revealed that HCQ usage was independently associated with a reduced risk of irreversible organ damage (overall and in the cutaneous and renal systems) and improved survival in SLE patients.[12,14]

In our selected SLE patients, IL-6, IP-10, sCD40L, IFN-α, TNF-α, MCP-1 and IL-1β were elevated compared with levels in control patients. Some of those cytokines have been associated with thrombosis in SLE and APS patients.[21,22] It is interesting to note that over two-thirds of the selected patents were positive for aPL antibodies. Soluble CD40 ligand, IL-6, IFN and IFN-regulated cytokines such as IP-10 and MCP1 have all been associated with increased disease activity in lupus patients.[4–7] However, only IFN-α was significantly associated with SLAM-R scores in our study. Perhaps an important consideration is that there are many correlates of disease activity in addition to disease activity scores such as complement levels, dsDNA antibodies and inflammatory markers like ESR that were not evaluated in this study. However, IFN-α is regarded as a signature cytokine in lupus patients, playing a key role in the immune dysfunction that characterizes the disease. An elevated level of this cytokine was one of the first and most extensively documented cytokine abnormalities in SLE, being associated with disease activity, disease severity, immune activation and several clinical features.[23]

An important mechanism of HCQ action is the inhibition of toll-like receptor activation, which has generally been attributed to its inhibition of endosomal acidification, a prerequisite of endosomal TLR activation, since it is a weak base that can partition into acidic vesicles.[24] The result is decreased inflammatory cytokine production and antigen processing necessary for antigen presentation of autoantigens. A recently published study has provided evidence suggesting that the direct binding of antimalarial drugs to nucleic acids, masking their TLR-binding epitopes, is the mechanism which prevents TLR

activation.[25] Circulating DNA and RNA-containing immune complexes in the blood of SLE patients activate plasmacytoid dendritic cells (pDCs) through TLR9 and TLR7, resulting in the production of proinflammatory cytokines, particularly IFN-α, and disease development. [26] The significant association of the reduction of IFN-α as a result of HCQ therapy with the decrease in SLAM-R shown in this study indicates that the inhibition of TLR activation resulting in decreased proinflammatory cytokine production and antigen presentation may be of paramount importance in the beneficial effect of HCQ therapy in SLE patients. In fact, anti-IFN monoclonal antibody therapy in a phase I trial resulted in clinical improvement in SLE patients, adding credence to the important role played by correction of this cytokine abnormality in the treatment of disease.[27] Additionally, there is in-vitro evidence that HCQ therapy induces apoptosis in lymphocytes and endothelial cells, prevents calcium dependent signaling in T cells and inhibits the secretion of several other cytokines in PBMCs, including IL-1, IL-6 and TNF-α.[28–32] The reduction of IL-6, TNF-α and sCD40L levels seen as a result of HCQ therapy in our study, although having no association with the clinical improvement associated with treatment, may suggest a possible secondary role for some of these additional mechanisms.

In addition, HCQ possesses an anti-thrombotic effect by inhibiting platelet aggregation and arachidonic acid release from stimulated platelets and our group has shown that HCQ inhibits aPL-induced platelet GPIIb/IIIa receptor expression in a dose dependent fashion but does not seem to affect TF-related pathways.[33,34] Edwards et al. demonstrated a dose-dependent relationship between HCQ treatment and decreased thrombus size and total time of thrombus formation in aPL-injected mice.[15] More recently, Rand et al. have demonstrated that HCQ reverses the binding of aPL–β_2GPI complexes to phospholipid bilayers and protects the annexin A5 anticoagulant shield from disruption by aPL.[35,36] In a Cox multiple failure time analysis, antimalarial drugs were shown to protect against thrombosis and increase survival in SLE patients and findings of a cross-sectional study by Erkan et al. suggested that HCQ was protective against thrombosis in aPL-positive individuals. [37,38] Multivariate analyses of several large lupus cohorts demonstrated a reduced risk of thrombosis with HCQ therapy. Interestingly, this was not the case for the LUMINA cohort of patients in whom HCQ was shown to provide no protection against the development of thrombosis. [39–42] Further studies in lupus cohorts consisting of patients of diverse ethnic backgrounds would clarify this point. McCarty and Cason previously reported that aCL titers decrease in patients treated with HCQ and aspirin and perhaps this also has some bearing on how HCQ prevents thrombosis in SLE/APS patients.[43] However, conflicting data have been obtained from subsequent studies; Erkan et al. found that there was no correlation between HCQ treatment and change in aCL titers in a large cohort of aPL-positive patients.[44] Similarly, in our patients, HCQ had no effect on the median of aCL titers.

A potential limitation of the study is that the small number of patients selected due to strict inclusion and exclusion criteria may have prevented several of the observed comparisons attaining statistical significance. Furthermore, there were several patients that met inclusion criteria but could not be included due to insufficient volumes of stored serum and/or plasma samples, which is unsurprising since some of these have been stored and tested for over 10 years.

Despite these potential limitations, we have shown that HCQ therapy results in significant clinical improvement in SLE patients as measured by reductions in SLAM-R scores. We also demonstrated that the decreases in SLAM-R in response to HCQ therapy are strongly correlated with reductions in IFN-α levels, indicating an important role for the inhibition of endogenous TLR activation in the beneficial effects of HCQ therapy in SLE patients. Our study provides additional evidence implicating type I interferons as an important factor in

disease progression in SLE patients and that therapies targeting the IFN pathway may be effective in modulating disease activity.

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Table 1

Clinical characteristics of the SLE patients included in this study

Disease characteristic*	Frequency (%)
ACR criteria	
• ANA positive	35/35(100.0)
• Malar rash	28/35 (80.0)
• Discoid rash	12/35 (34.3)
• Photosensitivity	27/35 (77.1)
• Ulcers	24/35 (68.6)
• Arthritis	28/35 (80.0)
• Serositis	23/35 (65.7)
• Neuropsychiatric	9/35 (25.7)
• Renal	18/35 (51.4)
• Hematological	33/35 (94.3)
• Immunological	30/35 (85.7)
	Mean ± SD
Disease duration	9.7 years ± 3.9
SLICC Damage Index	2.5 ± 1.9
SLAM-R	6.2 ± 5.1

*ACR criteria recorded in a cumulative manner at the last study visit.

Table 2

Effect of hydroxychloroquine (HCQ) therapy in SLE patients on biomarker levels and disease activity scores

	HCQ therapy		
Biomarker	**Before Rx/median**	**After Rx/median**	***p*-value**
IL-6 (pg/ml)	10.68	5.79	0.7956
IL-8 (pg/ml)	22.27	16.37	0.9390
MCP-1 (pg/ml)	665.96	738.97	0.5361
IP-10 (pg/ml)	525.85	556.81	0.7913
sCD40L (pg/ml)	3053.52	1241.83	0.9027
IFN-α (pg/ml)	573.06	381.03	0.2507
IL-1β (pg/ml)	0.00	0.00	0.2645
TNF-α (pg/ml)	9.10	7.55	0.8663
aCL-IgG (GPL)	9.09	9.60	0.5996
aCL-IgM (MPL)	3.04	3.28	0.8870
aCL-IgA (APL)	0.12	0.11	0.9096
SLAM-R	**9**	**7**	**0.0157**

The aCL results were expressed in GPL (for IgG aCL) and MPL (for IgM aCL) units, defined as the cardiolipin binding activity of one microgram per milliliter of an affinity-purified IgG or IgM preparation from a standard serum.