ORIGINAL ARTICLE

The effect of hydroxychloroquine on lupus erythematosus-like skin lesions in MRL/lpr mice

Tatsuya Shimomatsu1,2, Nobuo Kanazawa1, Naoya Mikita1, Yumi Nakatani1, Hong-jin Li1, Yutaka Inaba1, Takaharu Ikeda1, Toshikazu Kondo3, and Fukumi Furukawa1

1Department of Dermatology, Wakayama Medical University, Wakayama, Japan, 2Department of Dermatology, Wakayama Rosai Hospital, Wakayama, Japan, and 3Department of Forensic Medicine, Wakayama Medical University, Wakayama, Japan

Abstract

Objectives: To evaluate the effect and safety of hydroxychloroquine (HCQ) on lupus erythematosus (LE)-like skin lesions in the MRL/lpr mouse, a model for systemic LE (SLE).

Methods: We divided the MRL/lpr mice into three groups that were given: (1) drinking water, (2) HCQ at a dose of 4 mg/kg/d, or (3) HCQ at a dose of 40 mg/kg/d. The HCQ was administered to examine the effect and safety of HCQ on skin lesions and the number of infiltrating cells including mast cells in the dermis.

Results: Six of 13 mice in the group given drinking water, 3 of 11 mice in the group administered low-dose HCQ (4 mg/kg/d), and 1 of 10 mice in the group administered high-dose HCQ (40 mg/kg/d) presented the skin lesions. The average number of mast cells was 81, 50, and 12 (magnification, ×100), the mortality rate was 24%, 8%, and 9% and the mean body weight gain was 4.6 g, 8.0 g and 5.1 g, respectively.

Conclusions: HCQ was demonstrated to decrease the appearance of LE-like lesions and the number of mast cells in the dermis. Furthermore, there were no obvious systemic adverse effects. This study provides evidence that suggests benefits in human patients.

Keywords
Hydroxychloroquine, Mast Cell, MRL/Lpr mice; Skin lesion, Systemic lupus erythematosus

History
Received 8 June 2015
Accepted 8 January 2016
Published online 7 March 2016

Introduction

Although antimalarial drugs, such as chloroquine (CQ) and hydroxychloroquine (HCQ) were originally developed for the treatment of malaria, they can also be beneficial for inflammatory diseases including many dermatological, immunological, and rheumatological diseases. HCQ is derived from chloroquine β-hydroxylation and was first synthesized during the 1950s [1–3]. CQ and HCQ do not differ in terms of their mechanism of action, pharmacokinetics, toxicology, adverse effect, and indications [1]. Nowadays antimalarial drugs, especially HCQ, are acknowledged worldwide to be the first-line drugs in systemic therapy for the treatment of cutaneous lupus erythematosus (CLE) [4–7].

However, Japanese physicians cannot prescribe antimalarial drugs to CLE patients because of previous medical accidents that resulted in severe retinopathy due to excessive daily dosages. The production of antimalarial drugs was suspended in 1974 [8].

As underlying mechanisms for the clinical effects of HCQ, it has been shown that HCQ can suppress the production of prostaglandins and inflammatory cytokines and regulate the innate immune system [1–3]. Furthermore, in systemic lupus erythematosus (SLE), HCQ is reportedly associated with impaired interferon-α (IFN-α) and tumor necrosis factor-α (TNF-α) production by plasmacytoid dendritic cells (pDCs) [9].

However, the precise mechanisms by which HCQ exerts its effect on CLE, especially in vivo, remain to be defined. Here, we report that HCQ was administered to MRL/lpr mice, which are the animal model for SLE, showing immune complex glomerulonephritis, arteritis, arthritis, anti-DNA antibody and spontaneous lupus erythematosus (LE)-like lesions with Immunoglobulin G (IgG) deposits at the dermoepidermal junction [10,11]. We investigated the mechanism by which HCQ affected the development of the LE-like skin lesions in MRL/lpr mice, and examined systemic side effects including renal disease, weight change, and mortality.

Materials and methods

Mice

MRL/lpr mice were purchased from Japan SLC Inc. (Hamamatsu, Japan) and bred in individual cages. All experiments were performed in accordance with our institutional guidelines.

We administered HCQ (Sanofi-aventis, Meyrin/GE, Switzerland) orally to the MRL/lpr mice for four months from three months of age. We determined concentration of HCQ by calculating the average amount of drinking water a day and body weight, and administered the mice using feeding bottle.

The MRL/lpr mice were divided into the following three groups that were given: (1) drinking water (filtered water); vehicle control, (2) HCQ at 4 mg/kg/d, and (3) HCQ at 40 mg/kg/d. HCQ was dissolved in filtered water. The lower dose of HCQ (4 mg/kg/d) is about what is normally given to human patients.
Light microscopic observation of the skin and kidney

At four months after the first administration of HCQ, mice were sacrificed by cervical dislocation, and skin specimens were taken from the upper back regions of all mice. All specimens were fixed in 4% formaldehyde buffered with phosphate-buffered saline (PBS) (pH 7.2), embedded in paraffin, and stained with hematoxylin and eosin (HE) and toluidine blue (TB). This staining was performed to evaluate the number of infiltrating mast cells in the dermis. For the assessment of dermal infiltrating cells, we randomly selected five microscopic fields (original magnification, ×100), and counted the number of cells in each microscopic field. The number of infiltrating cells was expressed as the average number of mast cells in the five microscopic fields according to previously described methods [12]. All measurements were performed without prior knowledge of the experimental procedures.

Kidney specimens of all mice were subjected to HE and periodic acid-Schiff (PAS) staining. Pathological changes were evaluated and graded according to previously reported methods [13].

Proteinuria

Urine samples were collected at four months after the first administration to MRL/lpr mice, and protein contents were evaluated using ALBUSTIX urine test paper (Miles-Sankyo Co., Ltd, Tokyo, Japan) according to previously described methods [12]. Proteinuria was evaluated as follows: 0 (0–30 mg/dl), 1 (30–100 mg/dl), 2 (100–300 mg/dl), and 3 (300–1000 mg/dl).

Immunofluorescence method

To investigate the immunoglobulin deposits in the skin, skin specimens were taken from upper back region and embedded in Tissue-Tek O.C.T compound (Sakura Finetek Japan Co., Ltd, Tokyo, Japan) quickly frozen in liquid nitrogen, and stored at −80°C for subsequent immunoperoxidase staining. Frozen sections were cut using a cryostat CM1900 (Leica Microsystems, Heidelberg, Germany). The cryosections were immunostained with Alexa Fluor® 488-conjugated antimouse IgG (Invitrogen, Eugene, OR).

Antinuclear antibody

Serum samples were collected at four months after the first administration of HCQ, and we analyzed the presence and intensity of antinuclear antibody (ANA) staining in sera using a commercial kit (Fluoro HEPANA test, MBL Co., Nagoya, Japan).

Anti-dsDNA antibody

Anti-dsDNA antibody was measured in each serum sample by ELISA using mouse anti-dsDNA IgG (Alpha Diagnostic Intl. Inc., San Antonio, TX).

The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, blood urea nitrogen

Sera were collected to determine the serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, blood urea nitrogen with a Fuji DRI-CHEM 5500V (Fuji Medical System, Tokyo, Japan), according to the manufacturer’s instructions [14].

Expression of mRNA

The levels of mRNA for IL-2, IL-10, IL-12, TNF-α, and IFN-α were determined by reverse transcription–polymerase chain reaction (RT–PCR) analysis using the skins from the upper back of all mice. All RNA was extracted from dorsal skin specimens with Sepazol®-RNA (Nacalai Tesque, Inc., Kyoto, Japan). Single-stranded cDNA was reverse-transcribed using each RNA sample and amplified by PCR using Ex Taq (TAKARA Bio Inc., Otsu, Japan). The primer pairs used are shown in Supplementary Table 1. Then, the electrophoresed PCR products in 2% agarose gels were stained with ethidium bromide. The density of the bands was measured with Adobe Photoshop CS4 software (Adobe Systems Inc., San Jose, CA), and the data were normalized using a β-actin reference.

Weight change and mortality

The weight of each mouse was measured every month and mortality was estimated during the experimental period.

Statistical analysis

For comparisons of the frequency of LE-like skin lesions and mortality, Fisher’s exact probability test was used. Student’s t-test was employed for comparisons of mast cell recruitment, proteinuria, weight change, the serum levels of ANA, AST, ALT, creatinine, blood urea nitrogen, and the mRNA level of each cytokine. p Values <0.05 were regarded as statistically significant.

Results

Effects of HCQ administration on the development of LE-like skin lesions and dermal infiltration of mast cells

As shown in Table 1, 6 of 13 mice in the group given drinking water, 3 of 11 mice in the group administered low-dose HCQ (4 mg/kg/d), and 1 of 10 mice in the group administered high-dose HCQ (40 mg/kg/d) presented skin lesions. As shown in Figure 1(c), pathological LE-like skin lesions of MRL/lpr mice drinking water showed characteristic histopathological alterations such as acanthosis with hyperkeratosis, lymphocyte infiltration into the dermis and vasodilatation in the upper dermis. There was a pathologically significant difference between the group drinking water and the group administered high-dose HCQ (40 mg/kg/d) by Fisher’s exact probability test (p <0.05) (Table 1). These observations implied that HCQ decreased the development of spontaneous LE-like skin lesions in MRL/lpr mice.

As shown in Figure 1(e), histopathological changes associated with the skin lesions of MRL/lpr mice consisted of numerous mast cells infiltrating the dermis. However, in skin with no lesion, there were few mast cells in the dermis (Figure 1f). Figure 2 shows that the number of mast cells in the group drinking water was significantly elevated compared with the number in the group administered high-dose HCQ (40 mg/kg/d) by Student’s t-test (p <0.05). Regarding the immunofluorescence study, there was no significant difference in IgG deposits in the skin among the three groups (data not shown).

Table 1. The frequency of spontaneous LE-like skin lesions in MRL/lpr mice.

| Groups               | Skin lesions, n (%)* |
|----------------------|----------------------|
|                      | –        | +        | ++       |
| Control (n = 13)     | 7 (53.8) | 1 (7.8)  | 5 (38.5) |
| HCQ (n = 21)         | 17 (81.0)| 2 (9.5)  | 2 (9.5)  |
| 4 mg/kg/d (n = 11)   | 8 (72.7) | 1 (9.1)  | 2 (18.2) |
| 40 mg/gld (n = 10)   | 9 (90.0) | 1 (10.0) | 0 (0)    |

* Fisher’s exact probability test (p <0.05).
+: Only macroscopic skin lesions or pathological changes.
++: Both macroscopic skin lesions and pathological changes.
Effects of HCQ administration on the mRNA expression of cytokines

We determined the mRNA expression of IL-2, IL-10, IL-12, TNF-α, and IFN-α by RT–PCR analyses. There was no significant difference for all cytokines in the level of expression among the control group and the groups administered HCQ (4 mg/kg/d and 40 mg/kg/d). In the group given drinking water, the expression IL-2 was higher than the level in the groups administered HCQ, but the difference was not statistically significant (Supplementary Figure 1).

Effects of HCQ administration on SLE-related and other traits

There was no significant difference in the degree of proteinuria in the MRL/lpr mice among three groups (Supplementary Figure 2). The same held true for the degree of glomerulonephritis in the MRL/lpr mice with no significant difference, in renal histopathological alterations among the three groups (data not shown).

The mortality rate was 24% in the group given water, 8% in the group given low-dose HCQ (4 mg/kg/d), and 9% in the group given high-dose HCQ (40 mg/kg/d). There were no significant differences among the three groups (Supplementary Table 2).
From three months to seven months of age, the mean body weight gain was 4.6 g, 8.0 g, and 5.1 g in the drinking water, low-dose HCQ, and high-dose HCQ groups, respectively. The differences were not statistically significant.

Regarding the serum levels of AST, ALT, creatinine, and blood urea nitrogen, there were no significant differences among the three groups (Supplementary Table 3).

Similarly, there was no significant difference in the serum ANA levels or anti-dsDNA antibody titers among the three groups (data not shown).

Discussion

Antimalarial drugs were developed primarily to treat malaria but, they have become beneficial in the treatment of inflammatory disease. They are arguably the best modality currently available for treating patients with CLE. However, the precise mechanism by which they provide therapeutic effect is not well defined.

In this study, we focused on the effectiveness of HCQ for LE-like skin lesions in MRL/lpr mice, and investigated the prevalence of systemic side effects.

Our study demonstrated that HCQ decreased the development and severity of spontaneous LE-like skin lesions in MRL/lpr mice. Furthermore, the number of mast cells in the dermis of the upper skin regions of the group administered high-dose HCQ (40 mg/kg/d) was significantly decreased compared with the number in the group given water only. Previously, our group demonstrated that UVA1 irradiation might induce the apoptosis of mast cells in the dermis of MRL/lpr mice and consequently reduce the number of infiltrating mast cells in the dermis, as a partial explanation of the mechanism of skin lesion development in HCQ. In addition, HCQ might also be effective when injected into skin lesions of MRL/lpr mice in small doses instead of being administered in high dose.

HCQ can also suppress several inflammatory cytokines [2, 3, 19]. Previous reports detected significantly more expression of IL-2, IL-10, IL-12, and TNF-α mRNAs in the lesional skin of MRL/lpr mice, compared with the non-lesional skin from the same mouse by RT–PCR [20]. We analyzed the mRNA expression of these cytokines and IFN-γ also by RT–PCR. IFN-γ can be produced in SLE patients by pDCs in response to continuous stimulation by Toll-like receptors (TLR-9 and TLR-7), which is reported as a central contributor to the pathogenesis of SLE [9]. HCQ was demonstrated to be of therapeutic benefit in SLE by several mechanisms, one of which is inhibiting the production of IFN-γ in pDCs by blocking Toll-like receptors’ (TLR-9 and TLR-7) stimulation [21, 22]. As a result, there were no significant changes in the levels of any of the cytokines between the group given drinking water and the groups given HCQ (4 mg/kg/d or 40 mg/kg/d). It was found that the density of IL-2 in the group given drinking water was the highest as compared with the groups administered HCQ, but the difference was not statistically significant. Th1 cytokines, characterized by the expression of IL-2 and IFN-γ, may be critically important for the induction, development, and maintenance of chronic cutaneous lupus erythematosus [20, 23]. IL-2 in the skin of MRL/lpr mice may possibly accelerate or correlate with the severity of the skin disease [20]. Further, antimalarial drug (CQ) inhibits the production of IL-2 by interfering with the production of IL-2 mRNA, and also inhibits T-cell proliferation by interfering with IL-2 production [24]. In this study, we demonstrated that the density of IL-2 was higher in the group given drinking water than in the groups administered HCQ. Therefore, it is suggested that HCQ affects the development of LE-like skin lesions in MRL/lpr mice by the suppression of IL-2 expression (Supplementary Figure 3).

Regarding the systemic effect of HCQ in MRL/lpr mice, HCQ had no significant effect on proteinuria, renal disease, weight change, mortality rate, the serum levels of AST, ALT, creatinine, blood urea nitrogen, serum level of ANA, or anti-dsDNA antibody titer. Although we did not examine the eyes of MRL/lpr mice for retinopathy, no mice in the group administered HCQ (4 mg/kg/d or 40 mg/kg/d) showed abnormal behavior that seemed to be due to visual impairment. We did not examine cardiac function.

Conclusion

In this study, we demonstrated that HCQ decreased the development of LE-like skin lesions in MRL/lpr mice. This study suggested that HCQ suppress the infiltration of mast cells and the expression of IL-2 in the dermis, as a partial explanation of the mechanism of skin lesion development in HCQ. In addition, HCQ
did not have obvious systemic side effects in the examined dose range. This mouse study provides a better understanding of the effects of HCQ on CLE and supports potential benefits in human patients.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

References

1. Ochsendorf FR. Use of antimalarials in dermatology. J Dtsch Dermatol Ges. 2010;8:829–45.
2. Wolf R, Wolf D, Ruocco V. Antimalarials: unapproved uses or indications. Clin dermatol. 2000;18:17–35.
3. Kalla S, Dutz JP. New concepts in antimalarial use and mode of action in dermatology. Dermatol Ther. 2007;20:e:160–74.
4. Kuhn A, Ruland V, Bonsmann G. Cutaneous lupus erythematosus: update of therapeutic options part I. J Am Acad Dermatol. 2011;65:e179–93.
5. Chang AY, Werth VP. Treatment of cutaneous lupus. Curr Rheumatol Rep. 2011;13:300–7.
6. Yokogawa N, Kato Y, Sugii S, Inada S. Response to hydroxychloroquine in Japanese patients with systemic lupus erythematosus using the cutaneous lupus erythematosus disease area and severity index (CLASI). Mod Rheumatol. 2012;22:249–55.
7. Ikeda T, Kanazawa N, Furukawa F. Hydroxychloroquine administration for Japanese lupus erythematosus in Wakayama: a pilot study. J Dermatol. 2012;39:531–5.
8. Furukawa F. Practical therapeutics for skin lesions of Japanese patients with discoid lupus erythematosus. Expert Opin Orphan Drugs 2014;2(5):477–82.
9. Sacre K, Criswell LA, McCune JM. Hydroxychloroquine is associated with impaired interferon-alpha and tumor necrosis factor-alpha production by plasmacytoid dendritic cells in systemic lupus erythematosus. Arthritis Res. 2012;14:R155.
10. Theoﬁlopoulos AN, Dixon FJ. Etiopathogenesis of murine SLE. Immunological Rev. 1981;55:179–216.
11. Furukawa F, Tanaka H, Sekita K, Nakamura T, Horiguchi Y, Hamashima Y. Dermatopathological studies on skin lesions of MRL mice. Arch Dermatol Res. 1984;276:186–94.
12. Mikita N, Kanazawa N, Yoshimasu T, Ikeda T, Li H, Yamamoto Y, et al. The protective effects of ultraviolet A1 irradiation on spontaneous lupus erythematosus-like skin lesions in MRL/lpr mice. Clin Dev Immunol. 2009;2009:673952.
13. Nakatani K, Fujii H, Hasegawa H, Terada M, Arita N, Ito M, et al. Endothelial adhesion molecules in glomerular lesions: association with their severity and diversity in lupus models. Kidney Int. 2004;65:1290–300.
14. Kimura A, Ishida Y, Wada T, Hisaoka T, Morikawa Y, Sugaya T, et al. The absence of interleukin-6 enhanced arsenite-induced renal injury by promoting autophagy of tubular epithelial cells with aberrant extracellular signal-regulated kinase activation. Am J Pathol. 2010;176(1):40–50.
15. Yoshimasu T, Kanazawa N, Mikita N, Furukawa F. The expression of histamine receptors in skin lesions of MRL/Mp-lpr/lpr mice. Open Dermatol J. 2008;2:83–6.
16. Ito T, Seo N, Yagi H, Ohtani T, Tokura Y, Takigawa M, et al. Unique therapeutic effects of the Japanese-Chinese herbal medicine, Sairei-to, on Th1/Th2 cytokines balance of the autoimmunity of MRL/lpr mice. J Dermatol Sci. 2002;28(3):198–210.
17. Jabs DA, Lee B, Burek CL, Saboori AM, Prendergast RA. Cyclosporine therapy suppresses ocular and lacrimal gland disease in MRL/Mp-lpr/lpr mice. Invest Ophthalmol Vis Sci. 1996;37(2):377–83.
18. Everett MA, Coffey CM. Intradermal administration of chloroquine for discoid lupus erythematosus and lichen sclerosus et atrophicans. Arch Dermatol. 1961;83:977–79.
19. Willis R, Seif AM, McGwin JrG, Martinez-Martinez LA, Gonzalez EB, Dang N, et al. Effect of hydroxychloroquine treatment on pro-inﬂammatory cytokines and disease activity in SLE patients: data from LUMINA (LXXV), a multiethnic US cohort. Lupus 2012;21:830–5.
20. Nishide T, Yoshimasu T, Ikeda T, Seo N, Ohtani T, Furukawa F. Cytokine proﬁles of skin lesions in murine lupus models. J Dermatol Sci. 2005;1:537–43.
21. Boule MW, Broughton C, Mackay F, Akira S, Marshak-Rothstein A, Riklin IR. Toll-like receptor 9-dependent and –independent dendritic cell activation by chromatin-immunoglobulin G complexes. J Exp Med. 2004;199(12):1631–40.
22. Lövgren T, Eloranta ML, Kastner B, Wahlren-Herlenius M, Alm GV, Rönnblom L. Induction of interferon-alpha by immune complexes or liposomes containing systemic lupus erythematosus autoantigen-and Sjögren’s syndrome autoantigen-associated RNA. Arthritis Rheum. 2006;54:1917–27.
23. Toro JR, Finlay D, Dou X, Zheng SC, LeBoit PE, Connolly MK. Detection of type I cytokines in discoid lupus erythematosus. Arch Dermatol. 2000;136(12):1497–501.
24. Landewé RB, Miltenburg AM, Verdonk MJ, Verweij CL, Breedveld FC, Daha MR, et al. Chloroquine inhibits T cell proliferation by interfering with IL-2 production and responsiveness. Clin Exp Immunol. 1995;102:144–51.

Supplementary material available online
Supplementary Tables 1 and 2;
Supplementary Figures 1–3.