Original Paper

Ginsenoside Rh2 Suppresses Neovascularization in Xenograft Psoriasis Model

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Key Words

Ginsenoside Rh2 (GRh2) • Psoriasis • Soluble Flt-1 (sFlt1) • Neovascularization

Abstract:

Background/Aims: Psoriasis is a common inflammatory skin disease of undetermined etiology and poor prognosis. The current therapies have focused on direct inhibition of local inflammation, e.g. through hormone treatments. However, neovascularization plays a critical role in the development of psoriasis but so far no therapies have been developed to suppress psoriasis-associated neovascularization. **Methods:** We treated AGR129 mice that had received human PN skin grafts with different doses of Ginsenoside Rh2 (GRh2). The acanthosis and papillomatosis index were evaluated. The percentage of T lymphocytes in the grafts was quantified by flow cytometry. The levels of vascularization in the grafts were quantified based on CD31-positive area. We examined the levels of VEGF-A in the skin treated with GRh2. We treated AGR129 mice that had received human PN skin grafts with different doses of soluble Flt-1 (sFlt1) and then evaluated the effects on the acanthosis and papillomatosis index, T lymphocyte percentage and vessel density. **Results:** GRh2 dose-dependently decreased the acanthosis and papillomatosis index, T lymphocyte percentage and vessel density in PN skin grafts in mice. GRh2 inhibited VEGF-A levels in the PN skin grafts. Treatment with sFlt1 mimicked the effects of GRh2 on the acanthosis and papillomatosis index, T lymphocyte percentage and vessel density in PN skin grafts in mice. **Conclusions:** GRh2 may have an anti-psoriasis effect through neovascularization suppression.

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Introduction

Psoriasis is a common inflammatory skin disease of undetermined etiology, for which so far there is no cure [1-3]. Psoriasis is clinically characterized by prominent epidermal hyperplasia and a distinct inflammatory infiltrate [4]. Crosstalk between inflammatory cells and keratinocytes results in the production of cytokines, chemokines and growth factors, which mediate the development of the disease [5].

Mouse models have been combined with human cell xenografts and patient material are now applied to study the molecular mechanisms that underlie the development of inflammation in patients with psoriasis [1, 6, 7]. Hence, a combination of genetic and immunological investigations will help to develop advanced treatment of psoriasis and novel curative strategies.

Most patients of psoriasis develop a chronic 'plaque-type' psoriasis referred to as psoriasis vulgaris [8]. The prototypic skin lesions in these patients are erythematous, well-demarcated plaques covered by thick, whitish multilayered scales, which frequently detach [9]. In early lesions, the predominant dermal changes are tortuous, dilated vessels, papillar edema and perivascular infiltrates of dendritic cells (DCs), lymphocytes and macrophages [10]. In fully developed lesions, such as those in patients with plaque psoriasis, massive epidermal changes are observed with thickening of the epidermis (acanthosis), elongated epidermal rete ridges and, consequently, elongated dermal papillae containing dilated capillaries [11]. Histopathological features observed in human psoriasis are also observed in murine psoriasis-like disease [6, 12]. Therefore, mouse models have been extensively used to study human psoriasis.

The pathogenesis of psoriasis is complicated and so far not completely understood. During the development of psoriasis, the injured keratinocytes stimulates plasmacytoid dendritic cells (DC) to produce cytokines to promote DC activation, and vascular endothelial growth factor A (VEGF-A), which promotes vasodilatation and increased vascular permeability [1-3]. The VEGF family is composed of six secreted proteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PLGF) [13-15]. VEGF-A-mediated angiogenesis and neovascularization have been shown to be most important for embryonic and adult vessel formation and maintenance, in both physiological conditions and pathological conditions [16-20]. Once activated, DCs migrate into skin-draining lymph nodes to present an as yet unidentified antigen to naive CD4⁺ and CD8⁺ T cells and promote their differentiation, which produce and release cytokines to attract inflammatory cells to migrate into the skin via lymphatic and blood vessels to mediate the disease progress [21, 22]. The current therapies have focused on direct inhibition of local inflammation, e.g. through hormone treatments [21, 22]. However, although neovascularization plays a critical role in the development of psoriasis, so far no therapies have been developed to suppress psoriasis-associated neovascularization.

Ginsenoside Rh2 (GRh2) is a protopanaxadiol (PPD)-type ginsenoside (3 β -(β -D-Glucopyranosyl);R-form-Ginsenoside Rh2;dihydroxydammar-24-en-3-yl; β -D-Glucopyranoside, (3 β ,12 β)-12,20-;3 β -(β -D-Glucopyranosyloxy)dammar-24-ene-12 β ,20-diol;12 β ,20-Dihydroxy-5 α -dammar-24-en-3 β -yl β -D-glucopyranoside;b-D-Glucopyranoside, (3b,12b)-12,20-dihydroxydaMMar-24-en-3-yl) in red ginseng. The contents of GRh2 in red ginseng is about 0.015%, when prepared by treatment with steaming and air-drying [23]. However, its effects on the psoriasis have not been examined.

Here, we treated AGR129 mice that had received human prepsoriatic skin (PN skin) grafts with different doses of Ginsenoside Rh2 (GRh2). We found that GRh2 dose-dependently decreased the acanthosis and papillomatosis index, T lymphocyte percentage and vessel density in PN skin grafts in mice. Moreover, GRh2 inhibited VEGF-A levels in the PN skin grafts. Treatment with sFlt1 mimicked the effects of GRh2 on the acanthosis and papillomatosis index, T lymphocyte percentage and vessel density in PN skin grafts in mice.

Materials and Methods

Animals and patient specimen

All animal procedures in the current study have been approved and conducted by the Institutional Review Boards of in Shanghai Dermatology Hospital. AGR129 mice are deficient in type I (A) and type II (G) IFN receptors in addition to being RAG-2^{-/-}, and were purchased from Charles River Laboratories (China). Mice were kept pathogen free throughout the study and mice of 10 weeks of age were used for study.

Keratome biopsies (5X2X0.04 cm) of symptomless PN skin were taken from the lower back or buttock of patients with confirmed plaque-type psoriasis after informed consent was obtained. No topical or systemic medication was administered for at least 6 weeks before the study. PN skin from 8 different patients with confirmed plaque-type psoriasis was then transplanted onto AGR129 mice to allow the skin grafts to develop a psoriatic phenotype in the grafted mice. For the use of these clinical materials for research purposes, prior patient's consents and approval were collected, and the approvals by the Institutional Research Ethics Committee were obtained. Skin grafts were transplanted to the back of mice using an absorbable tissue seal.

Reagents

GRh2 (Weikeqi Bioscience, China) was prepared in a stock of 100mg/ml in normal saline and 100μl was injected subcutaneously into the skin grafts at 0.01mg/ml, 0.1mg/ml and 1mg/ml, respectively. sFlt1 (Becton-Dickinson Biosciences, San Jose, CA, USA) was prepared in a stock of 1μg/ml and 100μl was injected subcutaneously into the skin grafts at 0.1ng/ml, 1ng/ml and 10ng/ml, respectively.

Immunohistochemistry and flow cytometry

Acetone-fixed cryostat sections were stained using standard staining techniques. Unspecific Fc receptor binding of antibodies was measured with isotype-matched controls. Antibodies used were FITC-conjugated CD3 (Becton-Dickinson Biosciences) and unconjugated CD31 (Becton-Dickinson Biosciences). For analyses of T lymphocyte percentages, the skin grafts were dissected out and dissociated into single cells by 0.25% Trypsine (Sigma-Aldrich, St. Louis, MO, USA) for 45 minutes, passed through a 40μm filter and the incubated with FITC-CD3 or control antibody. Then the cells were sorted by Flow cytometry using a FACSAria (Becton-Dickinson Biosciences) flow cytometer.

Histologic Assessment and Quantification

Histologic quantification experiments represent the mean of three random fields with a 400-fold magnification. The acanthosis and papillomatosis index was defined as published [24]. The indicated values of both indices represent the mean of 10 random areas of each sample. Quantification of vessel density was based on CD31 staining, using NIH ImageJ software (Bethesda, MA, USA).

ELISA

The concentration of VEGF-A in the skin graft was determined by ELISA Kit (R&D System, Los Angeles, CA, USA). ELISA was performed according to the instructions of the manufacturer. Briefly, the extracted protein was added to a well coated with primary antibody, and then immunosorbed by biotinylated primary antibody at room temperature for 2 hours. The color development catalyzed by horseradish peroxidase was terminated with 2.5mol/l sulfuric acid and the absorption was measured at 450 nm. The protein concentration was determined by comparing the relative absorbance of the samples with the standards.

Statistical analysis

Statistical analyses were performed with SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). All data were statistically analyzed using one-way ANOVA with a Bonferoni correction, followed by Fisher's Exact Test to compare two groups. All values are depicted as mean ± standard deviation from 5 individuals and are considered significant if $p < 0.05$.

Results

Development of a psoriatic phenotype upon transplantation of PN Skin onto AGR129 Mice

PN skin from 8 different patients with confirmed plaque-type psoriasis was transplanted onto AGR129 mice. The skin grafts developed a psoriatic phenotype in 27 out of 30 (90%) grafted mice. Phenotypic conversion started at week 4 and was fully developed at 8 weeks after engraftment. Appearance of PN skin on the day of transplantation (Fig. 1A) was comparable to 8 weeks after transplantation onto AGR129 mice, in which PN skin grafts showed typical features of psoriasis (Fig. 1B). In summary, upon transplantation of PN skin onto AGR129 mice, resident skin cells including epidermal keratinocytes, DCs, endothelial cells, and immune cells became activated to create fully fledged psoriasis.

GRh2 dose-dependently suppresses psoriasis-like features

We injected 100µl GRh2 at different concentrations (0.01mg/ml, 0.1mg/ml and 1mg/ml) to the skin grafts in the mice. We found that GRh2 dose-dependently decreased the acanthosis and papillomatosis index (Fig. 2A). Moreover, GRh2 dose-dependently decreased T lymphocyte percentage by quantification (Fig. 2B), and by representative flow charts (Fig. 2C). Further, GRh2 dose-dependently decreased the vessel density in PN skin grafts in mice, by quantification (Fig. 2D), and by representative images (Fig. 2E). Together, these data suggest that GRh2 dose-dependently suppresses psoriasis-like features in skin grafts.

GRh2 dose-dependently inhibits VEGF-A levels in mice that received skin grafts

Since we found that GRh2 dose-dependently decreased the vessel density in PN skin grafts in mice, we were prompted to find out whether GRh2 may affect the levels of angiogenic factor VEGF-A. We isolated proteins from the skin grafts and used ELISA to determine the VEGF-A levels. We found that GRh2 dose-dependently inhibited VEGF-A levels in mice that received skin grafts (Fig. 3).

Inhibition of VEGF-A signaling suppresses psoriasis-like features

In order to find out whether GRh2 suppresses psoriasis-like features through inhibition of neovascularization, we used sFlt1 to treat skin grafts, which inhibits VEGF-A signaling through competitive receptor binding. We found that sFlt1 dose-dependently decreased the acanthosis and papillomatosis index (Fig. 4A). Moreover, sFlt1 dose-dependently decreased T lymphocyte percentage (Fig. 4B), and dose-dependently decreased the vessel density in PN skin grafts in mice (Fig. 4C). Together, these data suggest that GRh2 dose-dependently suppresses psoriasis-like features in skin grafts through inhibiting VEGF-A signaling (Fig. 5).

Fig. 1. Development of a psoriatic phenotype upon transplantation of PN Skin onto AGR129 Mice. (A-B) PN skin from 8 different patients with confirmed plaque-type psoriasis was transplanted onto AGR129 mice. The skin grafts developed a psoriatic phenotype in 27 out of 30 (90%) grafted mice. Phenotypic conversion started at week 4 and was fully developed at 8 weeks after engraftment. Appearance of PN skin on the day of transplantation (A) was comparable to 8 weeks after transplantation onto AGR129 mice, in which PN skin grafts showed typical features of psoriasis (B). In summary, upon transplantation of PN skin onto AGR129 mice, resident skin cells including epidermal keratinocytes, DCs, endothelial cells, and immune cells became activated to create fully fledged psoriasis. Scale bars are 100µm.

Fig. 2. GRh2 dose-dependently suppresses psoriasis-like features. We injected 100 μ l GRh2 at different concentrations (0.01mg/ml, 0.1mg/ml and 1mg/ml) to the skin grafts in the mice. (A) GRh2 dose-dependently decreased the acanthosis and papillomatosis index. (B-C) GRh2 dose-dependently decreased T lymphocyte percentage by quantification (B), and by representative flow charts (C). (D-E) GRh2 dose-dependently decreased the vessel density in PN skin grafts in mice, by quantification (D), and by representative images (E). (F) H&E staining. Statistics: one-way ANOVA with a Bonferoni correction, followed by Fisher's Exact Test to compare two groups. * $p < 0.05$. N=5. Scale bars are 50 μ m.

Discussion

In recent years, substantial advances have been made in elucidating the molecular mechanisms of psoriasis. However, major issues remain unresolved, including the primary nature of the disease as an epithelial or immunologic disorder, the autoimmune cause of the inflammatory process, the relevance of cutaneous versus systemic factors, and the role of

Fig. 3. GRh2 dose-dependently inhibits VEGF-A levels in mice that received skin grafts. We isolated proteins from the skin grafts and used ELISA to determine the VEGF-A levels. We found that GRh2 dose-dependently inhibited VEGF-A levels in mice that received skin grafts. Statistics: one-way ANOVA with a Bonferoni correction, followed by Fisher's Exact Test to compare two groups. * $p < 0.05$. $N = 5$.

Fig. 4. Inhibition of VEGF-A signaling suppresses psoriasis-like features. We used sFlt1 to treat skin grafts, which inhibits VEGF-A signaling through competitive receptor binding. (A) We found that sFlt1 dose-dependently decreased the acanthosis and papillomatosis index. (B) sFlt1 dose-dependently decreased T lymphocyte percentage. (C) sFlt1 dose-dependently decreased the vessel density in PN skin grafts in mice. Statistics: one-way ANOVA with a Bonferoni correction, followed by Fisher's Exact Test to compare two groups. * $p < 0.05$. $N = 5$.

Fig. 5. Schematic of the model. GRh2 may suppress psoriasis-like features in skin grafts through inhibiting VEGF-A signaling.

genetic versus environmental influences on disease initiation, progression, and response to therapy [1-4].

Most importantly, neovascularization has been shown to play a critical role in the pathogenesis of psoriasis, since it appears to be required for upgrades of inflammation, and inflammation-associated cytokine production and secretion [1-4]. Hence, therapeutic approaches targeting enhanced neovascularization may be a novel option for treating psoriasis.

In the current study, we examined the effects of GRh2 on the features of psoriasis. We found that GRh2 dose-dependently suppressed the features of psoriasis, including the acanthosis and papillomatosis index, T lymphocyte percentage and vessel density in PN skin grafts in mice. Hence, we focused on the effects of GRh2 on vessels, since the vascular pathology of psoriasis is relatively less investigated. Since VEGF-A is a major mediator of pathological neovascularization, we tried to use a VEGF-A antagonist to reproduce the effects

of GRh2. We selected sFlt1, since it is a decoy receptor for both VEGF-A and PLGF [16-20]. Since the levels of PLGF in the grafted skin are nearly undetectable, we think that sFlt1 may mainly target VEGF-A in this model. In a gain-of-function experiment, sFlt1 indeed mimicked the effects of GRh2 in a dose-dependent manner. Moreover, sFlt1 also inhibited the increases in T lymphocytes, which are pivotal players in the pathological development of psoriasis. Indeed, the activated T cells are a major component of the inflammatory infiltrate of psoriatic lesions. Further research has determined that T cells from patients with psoriasis could transmit disease in animal models [10]. These findings characterize the pathogenesis of psoriasis as immune mediated events in which skin-directed T cells play a central role. Once these pathogenic T cells have entered the skin, they become activated and release cytokines and chemokines to attract other immune cells to perpetuate the inflammatory cascade [11]. In our study, the suppression of pathological vessel formation may substantially reduce the infiltration of T cells, possibly through inhibition of vessel permeability. Hence, targeting neovascularization may have an effect on the T lymphocyte pathology in psoriasis.

Our study thus proposes a novel therapeutic strategy against psoriasis, through inhibiting inflammatory neovascularization. Future studies may be applied to further elucidate the related molecular changes in the GRh2-treated mice, which provides further evidence to apply the findings in this study to treat psoriasis patients.

Disclosure Statement

The authors have declared that no competing interests exist.

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