

Decreased Hydrogen Peroxide Generation by Neutrophils from Acne Patients Treated with Isotretinoin

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Background: Reactive oxygen species (ROS) generated by neutrophils are closely correlated with the pathogenesis of a variety of inflammatory skin diseases. The aim of this study was to investigate whether the amount of reactive oxygen species (hydrogen peroxide) generated by neutrophils from patients with acne inflammation decrease after oral administration of standard doses of isotretinoin.

Method: In order to measure neutrophil hydrogen peroxide production, phorbol myristate acetate (PMA, neutrophil stimulant), was added to whole blood. Intracellular dichloro-fluorescein (DCF) fluorescence of neutrophils was determined by flow cytometry. In order to assess treatment efficacy, we used a Global Acne Grading Score (GAGS) and assessed the efficacy based on examinations at baseline and week 8.

Results: Patients with acne inflammation showed a significantly increased level of hydrogen peroxide produced by neutrophils compared to healthy controls. Patients with acne inflammation treated with isotretinoin showed a significant decrease in the ability of neutrophils to produce hydrogen peroxide in accordance with a clinical improvement of acne lesions.

Conclusion: Our result shows that the generation of ROS which induce a chemical insult to the integrity of the follicular epithelium in acne, can be suppressed in isotretinoin-treated acne patients. (Ann Dermatol (Seoul) 18(2) 59~63, 2006)

Key Words: Isotretinoin, Hydrogen peroxide

INTRODUCTION

In the pathogenesis of acne inflammation, *Propionibacterium acnes* appears to play an important initiating role by producing low-molecular-weight chemotactic factors¹, which result in the accumu-

lation of neutrophils at the site of acne comedones. The attracted neutrophils, after phagocytosis, release inflammatory factors, such as lysosomal enzymes^{2,3} with resultant damage to the follicular epithelium. Hydrogen peroxide is one of the reactive oxygen species (ROS) primarily produced by phagocytic cells as a consequence of the process of phagocytosis⁴.

It has been reported that the ability of neutrophils to produce hydrogen peroxide was significantly increased in patients with acne inflammation⁵.

The aim of this study was to investigate whether the amount of hydrogen peroxide generated by neutrophils from patients with acne inflammation decreased after oral administration of standard doses of isotretinoin.

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MATERIALS AND METHODS

Reagents

2',7'-dichlorofluorescein diacetate (DCFH-DA) was purchased from Eastman Kodak, Rochester, NY. Flow Cytometry sheath solution (1.9 mmol/L potassium phosphate, monobasic and 139 mmol/L sodium chloride) stored at the room temperature, 100 mmol/L ethylene diamine tetra acetic acid (EDTA), 500 mmol/L sodium azide and Phobol Myristate Acetate (PMA). DCFH-DA and PMA was dissolved in DMSO at a concentration of 100 mmol/L, 1 mg/ml and stored in the dark at 4°C, -20°C.

Subjects

EDTA peripheral blood was obtained from twelve healthy adult volunteers and twelve patients with acne inflammation before treatment. In addition, EDTA peripheral blood was obtained from twelve patients with acne inflammation after 8 weeks treatment with isotretinoin (0.5 mg/kg per day)

Measurement of production of hydrogen peroxide

In order to measure neutrophil hydrogen peroxide production, PMA was added to whole blood. A mixture of 100 µL of EDTA whole blood, 2 µL of 100 mmol/L DCFH-DA in DMSO was incubated with rotational agitation for 15 min at 37°C in a shaking water bath, followed by the addition of 10 µL of 500 mmol/L sodium azide. The tube was incubated with rotational agitation for 15 min at 37°C in a shaking water bath, followed by the addition of 10 µL of PMA & EDTA. After incubation, the erythrocytes were removed by cold lysing solution for 10 min at room temperature and centrifuged with PBS, and each cell pellet was resuspended in 1.0 mL of 1.0% paraformaldehyde and analysed by flow cytometry.

Flow cytometry

Intracellular DCF fluorescence of neutrophils was determined by flow cytometry (FACS Analyzer, Becton Dickinson Immunocytometry Systems, CA) with 488 nm excitation. Green fluorescence was collected from DCF through a 530 nm band-pass

Table 1. Determination of Global Acne Grading System (GAGS) Global Grade

Location	Factor rate	Clinical assessment
Forehead	2	0 = no lesion
Rt. cheek	2	1 = ≥ 1 comedon
Lt. cheek	2	2 = ≥ 1 papule
Nose	1	3 = ≥ 1 pustule
Chin	1	4 = ≥ 1 nodule
Chest	3	

Sum of local scores = Global score

Global Grade	Global Score	Severity
0	0	None
1	1 - 18	Mild acne
2	19 - 30	Moderate acne
3	31 - 38	Severe acne
4	≥ 39	Very severe acne

filter in combination with a 570 nm dichroic mirror. In the final suspension, lymphocytes, monocytes, a few contaminating erythrocytes, aggregated cells, and debris were excluded from analysis using a gate analysis method. Quantification of hydrogen peroxide production was estimated from the mean DCF fluorescence per cell. At least 2000 neutrophils were examined in each sample.

Clinical efficacy

We used a Global Acne Grading System (GAGS)⁶ to assess clinical improvement of isotretinoin therapy (Table 1). The GAGS considers six locations on the face and chest/upper back, with a factor for each location based roughly on surface area, distribution, and density of pilosebaceous units. The borders on the face are delineated by the hairline, jawline, and ears. The chest and upper back have been included because their involvement is critical in order to assess the severity of acne and to decide upon treatment. Each of the six locations is graded separately on a 0 to 4 scale, with the most severe lesion within that location determining the local scores. At first visit we evaluated the GAGS of each patient and after 8 weeks treatment we reevaluated the GAGS.

Statistical evaluation

Statistical comparisons were determined using the unpaired Student's t-test when comparing patient data before treatment with patient data after treatment. Mean differences were considered to be significant when $p < 0.01$.

RESULTS

No patients were lost during follow-up. The male/female ratio was 7 males to 5 females, and the mean age was 20.8 years (range 12 to 45 years). The mean global score of the acne patient before and after treatment were 31.75 and 15.75, respectively ($p < 0.01$) (Fig. 1A). Patients with acne inflammation showed a significantly increased level of hydrogen peroxide produced by neutrophils compared to healthy controls. The amount of hydrogen peroxide generated by neutrophils from patients with acne inflammation was 132.75% of that generated by neutrophils from healthy controls. The amount of hydrogen peroxide generated by neutrophils from patients with acne inflammation decreased after oral administration of standard doses of isotretinoin ($p < 0.01$). The amounts of hydrogen peroxide generated by neutrophils from patients with acne inflammation before and after treatment were 132.75% and 110.50%, respectively, of that generated by neutrophils from healthy controls ($p < 0.01$) (Fig. 1B). Our result showed that the amounts of hydrogen peroxide generated by neutrophils decreased as similar to the normal control.

DISCUSSION

With respect to tissue injury, the term 'free radical' is used to characterize metabolites, usually of low molecular weight, which are considered to be reactive enough to damage essential biological molecules. Most of the 'free radicals' are derived from molecular oxygen. ROS normally exist in all

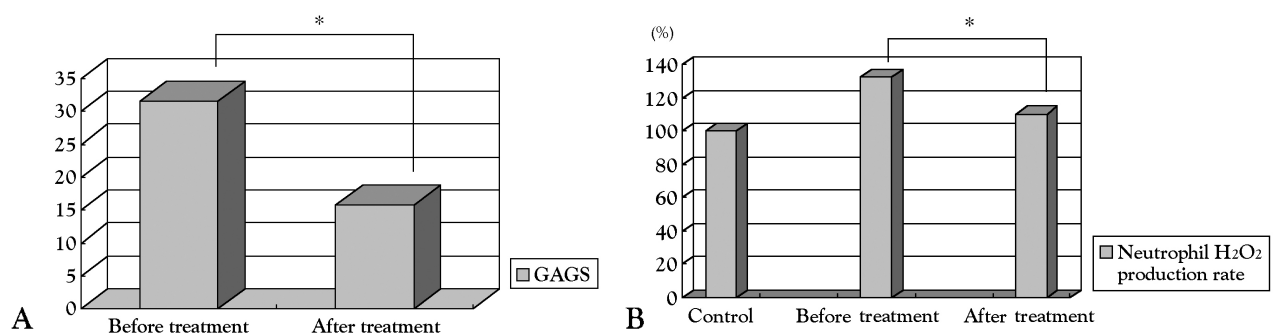


Fig. 1. (A) The mean global score of the acne patients before and after treatment were 31.75 and 15.75 $^{*}(p < 0.01)^{**}$. (B) Hydrogen peroxide generation by neutrophils from patients with acne inflammation before and after treatment with isotretinoin $^{*}(p < 0.01)$.

aerobic cells in balance with biochemical antioxidants. Oxidative stress occurs when this critical balance is disrupted because of excess ROS, antioxidant depletion, or both. Uncontrolled production of ROS leads to damage of biomolecules and may cause disease. There are now both direct and indirect evidence implicating ROS in many inflammatory skin disorders⁴. Production of ROS in skin may be caused by ionizing or UV radiation under aerobic conditions⁷; infiltration of PMNSs⁸; burn-induced deamination of adenine producing hypoxanthine acting as a substrate for xanthine oxidase⁹; photochemical production from pheomelanins; hyperbaric oxygen; photosensitizing agents (e.g., psolarens and porphyrins); phototoxicity and photoallergy; drug toxicity; aging; autoimmune disease; tumor promotion or enzymes; and coordination complexes containing transition metals in their lower oxidation states¹⁰.

Oral isotretinoin (13-cis-retinoic acid) has been used to treat acne vulgaris for more than 20 years. It significantly reduces the excretion of sebum¹¹, formation of comedones¹² and colonization of the skin with *P. acnes*^{11,13}, and also has anti-inflammatory activity¹⁴. Oral isotretinoin is currently the most effective acne treatment available, with reported long-term remission rates as high as 70-89%¹⁵⁻¹⁷. Treatment can be started at lower doses and increased subsequently according to tolerability¹⁹. However, published data indicates that the optimal benefit is achieved with a dose close to 1 mg/kg of body weight^{16,19,20}.

Systemic retinoids are effective in a variety of inflammatory dermatoses, disorders in which polymorphonuclear leukocytes (PMN) are involved, such as psoriasis and acne^{21,22}. In acne PMN invade the lower infundibulum of sebaceous follicles. Isotretinoin significantly inhibited monocyte and neutrophil chemotaxis and showed an anti-inflammatory effect in patients with acne conglobata²³. So we think that the ROS of the isotretinoin treated group could be decreased in our experiment. ROS induce a chemical insult to the integrity of the follicular epithelium in acne. So decreased ROS can induce clinical improvement. Patients with acne inflammation treated with isotretinoin showed a significant decrease in the ability of neutrophils to produce hydrogen peroxide in accordance with a clinical improvement of acne lesions. In a previous report, isotretinoin showed an antioxidant activity against

the superoxide anion²⁴. Our result also supports isotretinoin to have an inhibitory effect on the generation of ROS which has been reported to be capable of causing tissue injury, called auto-oxidative damage, at the sites of inflammation²⁵.

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