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Biochemical and immunological studies on erythrocytes superoxide dismutase modified by nitric oxide in patients with alopecia areata: Implications in alopecia patchy persistent and alopecia universalis

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ABSTRACT

Alopecia areata (AA) is a non-scarring hair loss disorder that ranges in severity from patchy loss of scalp hair (AA patchy persistent; AAP) to loss of all scalp and body hair (alopecia universalis; AU). The cause of AA is unknown but most evidences support that AA has an autoimmune etiology, where free radicals play an important role. This study was undertaken to investigate the role of nitric oxide (NO) modified erythrocytes superoxide dismutase (eSOD) in AA. Data revealed that NO-induced damage in eSOD caused alteration in hydrophobic or aromatic amino acids and protein carbonyl contents. NO-specific quencher, carboxyl-PTIO further reiterates NO-modifications. Specificity of antibodies from AA patients ($n = 26$) was analyzed toward NO-modified eSOD (NO-eSOD) and their results were compared with healthy controls ($n = 30$). Protein-A purified IgG of AA patients (AA-IgG) showed strong binding to NO-eSOD in comparison with IgG from controls. In addition, AA-IgG from patients with AU recognized NO-eSOD in a greater extent as compared to AA-IgG from patients with AAP. Furthermore, AU patients' sera contained higher levels of NO or carbonyl contents and lower levels of SOD activity compared with AAP patients' or control sera. In conclusion, this is the first study to demonstrate the role of NO-modified-eSOD in AA. Our novel results conclude that perturbations in SOD by NO presenting unique neo-epitopes that might be one of the factors for the antigen driven antibodies induction in AA. Preferential binding of NO-eSOD by AA-IgG pointed out the likely role of NO-eSOD in the initiation/progression of AA.

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1. Introduction

Alopecia areata (AA) is a common form of localized, non-scarring hair loss, autoimmune disorder. It ranges in severity from patchy loss of scalp hair (AA patchy persistent; AAP) to loss of all scalp and body hair (alopecia universalis; AU) [1]. Although the exact pathogenesis of AA is unknown, but the occurrence of autoimmune reactions have been assumed [2]. AA is now considered to be an autoimmune disease due to an aberrant T cells response against various self antigens [3]. This autoimmune etiology has been also proposed on the basis of its association with various autoimmune diseases [4], the presence of autoantibodies and various underlying immunologic abnormalities in the affected sites of these patients have also been reported [5], but the precise mechanism of generation of autoantibodies in AA remains unclear [5,6]. It is well documented that oxidative stress plays a vital role in AA as well as in other skin disorders [7–12]. Various studies have shown that AA is associated with increased formation of free radicals and decrease in antioxidant potential [7–10,13–15]. This may leads to oxidative damage of cell components including protein and nucleic acids as happened in various other autoimmune diseases [16–18].

Superoxide dismutase (SOD) is a prime antioxidant enzyme that destroys the effects of superoxide, thus limiting the deleterious effects of reactive oxygen and nitrogen species (RONS) [19]. Hence, SOD is considered an important regulator of oxidative/nitrosative stress. It is documented that dysfunctioning of SOD is reported in patients with AA [7–9,15] and it is also reported that it becomes antigenic in various chronic conditions [20]. Therefore, it is assumed that SOD may be continuously exposed to

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oxidative/nitrosative stress, so that alterations in conformation and function of SOD may occur, which may results in modification of its biological properties. In view of these, this study hypothesized that oxidative by-products, like nitric oxide (NO) damage erythrocytes SOD (eSOD), help to initiate autoimmunity in AA. To test this hypothesis, we studied the presence of circulating autoantibodies in AA patients directed against NO-modified eSOD (NO-eSOD) and to analysis their relationship in patients with AAP and AU. Our novel results not only support an association between NO-eSOD and AA, but also suggest that NO damaged eSOD may be an important biomarker for the evaluation in AA progression and in the elucidation of the mechanisms of disease pathogenesis.

2. Methods

2.1. Patients recruitment

This study has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki as revised in Tokyo 2004) for humans and was approved by local ethical comity of College of Medicine, Qassim University, KSA. Study subjects were recruited through the dermatology outpatient clinics of Qassim University, Buraidah, KSA and informed consent was obtained from each subjects. Patients were classified according to Alopecia Areata Foundation Clinical Assessment Guidelines [21]. The study group included 27 AA patients (5 female and 21 male) and their age range was 19–45 years (mean ± SD, 31.4 ± 7.32 years). The duration of the disease ranged from 1 month to 18 years. Patients with less than 100% scalp hair loss for more than or equal to 1 year were classified as AA, patchy persistent (AAP; *n* = 21). Patients with 100% scalp and body hair loss were classified as alopecia universalis (AU; *n* = 6). The control group comprised 30 healthy subjects (7 female and 23 male, age range 20–47 years, mean ± SD age 33.3 ± 10.8 years). The mean ages were not significantly different between the groups. The racial/ethnic and sex compositions of the AA groups were comparable with those of the control group. Venous blood samples from the control subjects and AA patients were collected and stored in small aliquots at −80 °C until analyzed further.

2.2. Modification of erythrocytes superoxide dismutase

Superoxide dismutase from erythrocytes (catalog # S5395, Sigma–Aldrich, St. Louis, MO, USA) was modified in Tris–HCl buffer (20 mM, pH 7.5) as previously described with slight modifications [22]. Briefly, an aqueous solution of eSOD (1 mg/ml) was modified by nitric oxide, generated by the reduction of sodium nitrite (100 mM) with sodium dithionite (10 mM). The modification was performed at 37 °C for 24 h and excess sodium nitrite or sodium dithionite were removed from the samples by extensively dialysis (dialysis tubing was from Sigma) against Tris–HCl buffer, pH 7.5.

2.3. Fluorescence measurements

Fluorescence measurements were performed on Anthos Zenyth 3100 Multimode Detectors (Salzburg, Austria). Different excitation and emission slits were set and intrinsic fluorescence was recorded by using native and NO-modified eSOD as described previously [20,23]. Protein samples (1 mg/ml) were also characterized specifically by hydrophobicity as described previously [24]. Briefly, native and NO-modified eSOD samples (1.5 μM) in 67 mM sodium phosphate buffer (pH 7.4) were probe with 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS) (10 μM) at 25 °C. The compound was excited at 394 nm and intrinsic fluorescence intensity was recorded. The decrease or increase of fluorescence intensity (FI) was calculated using the following equation.

$$\%\,\text{Decrease or increase of FI} = \left[\frac{FI_{\text{eSOD or NO-eSOD}} - FI_{\text{NO-eSOD or eSOD}}}{FI_{\text{eSOD or NO-eSOD}}}\right] \times 100$$

2.4. Assay for protein oxidation

Protein oxidation in protein samples or patients' sera was determined by carbonyl groups formation as previously described [25] with slight modifications. Briefly, the reaction mixture containing 15 μM of protein samples or 10 μl of serum samples, 0.5 ml of 10 mM 2, 4-dinitrophenylhydrazine (DNPH)/2.5 M HCl was added and thoroughly mixed. Nitrated proteins in the reaction mixture were precipitated by the addition of 20% (w/v) trichloroacetic acid (TCA, Sigma, MO, USA) and pellet was collected by centrifugation (Eppendorf Centrifuge, Hamburg, Germany). Ethanol and ethylacetate mixture (1:1 of 1 ml) was used to wash the pellet (3 times). The pellet was then dissolved in 1 ml of 6 M guanidine solution and was incubated for 15 min at 30 °C. After incubation, the reaction mixture was centrifuged and supernatant was collected for carbonyl contents estimation. Carbonyl contents were calculated by absorbance difference between test and control using the molar absorption coefficient of 22,000 M^{-1} cm^{-1} at 370 nm. Protein concentration was determined in the samples and carbonyl contents were expressed as nmol/mg protein.

2.5. Purification of immunoglobulin G

Immunoglobulin G (IgG) from human sera was isolated by affinity chromatography using Protein A-Agarose affinity column (cat. # PA1-EA, Sigma–Aldrich) as described previously [26]. Serum (0.3 ml) diluted with equal volume of PBS, pH 7.4 was applied to the column (12 mm × 45 mm) equilibrated with the same buffer. The flow through was reloaded onto the column 2–3 times. Unbound proteins were removed by extensive washing with PBS, pH 7.4. The bound IgG was eluted with 0.58% acetic acid in 0.85% sodium chloride and neutralized with 1.0 ml of 1.0 M Tris–HCl, pH 8.5, 1 ml fractions were collected and read at 251 and 278 nm. The IgG concentration was determined considering 1.38 OD_{278} = 1.0 mg IgG/ml [24]. The isolated IgG was dialyzed against PBS, pH 7.4 and stored at −20 °C.

2.6. Enzyme-linked immunosorbent assays

Direct binding ELISA was performed on flat bottom 96-well, polystyrene maxiSorp immunoplates (catalog # P8616; Nunc-Immuno™ MicroWell, Sigma–Aldrich) as described previously [27]. Briefly, polystyrene polysorp immunoplates were coated with 100 μl of native or modified eSOD (10 μg/ml) in carbonate buffer (0.05 M, pH 9.6). The plates were coated for 2 h at room temperature (RT) and overnight at 4 °C. Each sample was coated in duplicate and half of the plates served as control devoid of only antigen coating. Unbound antigen was washed with PBS-T (10 mM, 150 mM NaCl, pH 7.4 containing 0.05% Tween-20; Sigma, St. Louis, MO, USA) and unoccupied sites were blocked with block buffer (PBS containing 1% BSA) for 1–2 h at RT. After incubation, the plates were washed with PBS-T. The test purified IgGs in PBS (100 μl/well) was adsorbed for 2 h at RT and overnight at 4 °C. Bound antibodies were analyzed with anti-human HRP linked conjugate (catalog # sc2769, Santa Cruz Biotechnology, USA) using 3,3′,5,5′-tetramethylbenzidine substrate (TMB, catalog # 206697A, Santa Cruz Biotechnology). Reaction was stopped by stop solution (2 M H_2SO_4) and absorbance of each well was recorded at

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