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Original article

Non-invasive evaluation of atopic dermatitis based on redox status using *in vivo* dynamic nuclear polarization magnetic resonance imaging

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A B S T R A C T

Atopic dermatitis (AD) is a chronic inflammatory condition with complex etiology, including genetic, environmental and immunologic factors. Redox imbalance caused by excessive oxidative stress has been shown to mediate disease activity of AD. Currently, an imaging technique that can monitor the redox status of the skin *in vivo* has not yet been developed. Consequently, we have established such a technique that can detect and visualize the redox status of the skin using *in vivo* dynamic nuclear polarization magnetic resonance imaging (DNP-MRI). To evaluate this technique, we utilized an AD mouse model that was generated by repeated topical application of mite antigen in NC/Nga mice. We imaged alterations in redox balance of the resulting AD skin lesions of the mice. Using *in vivo* DNP-MRI and non-toxic nitroxyl radicals to visualize free radicals *in vivo*, we revealed that AD skin lesions demonstrated more rapid decay rates of image intensity enhancement than normal skin, indicating that our technique can monitor excessive oxidative stress occurring in AD skin lesions. Therefore, this technique has the potential to provide a novel approach for evaluating disease activity of inflammatory skin diseases, including AD, from the view point of altered redox status.

1. Introduction

Atopic dermatitis (AD) is an inflammatory skin disease that results from the interaction of genetic, environmental, and immunologic factors [1]. The dermis contains perivascular and interstitial infiltration of inflammatory cells such as plasma cells, mast cells, eosinophils, and B and T lymphocytes [2]. Recent studies have reported that oxidative stress may play an important role in many skin diseases including skin aging and AD [2–4]. Oxidants such as free radicals, reactive oxygen species (ROS), nitric oxide species are produced during normal metabolic activities. Biological antioxidant defense systems such as enzyme-based systems (superoxide dismutase, glutathione peroxidase, and peroxiredoxins) and non-enzyme-based systems (vitamins A, C, and E, glutathione, polyphenols, and coenzyme Q10) also exist in living organisms to oppose to these entities [2,5]. Severe oxidative stress in AD patients is reportedly induced by increased lipid peroxidation and a decrease in antioxidant levels [4,6], which suggests that suppression of oxidative stress could be a useful strategy for the treatment of AD [6–8]. It has also been demonstrated that oxidative stress promotes inflammation through upregulation of genes that encode proinflammatory cytokines in inflammatory cells [2] and in normal human keratinocytes [9]. Taken together, this evidence suggests that redox imbalance caused by severe oxidative stress and complex inflammatory responses plays an important role in the pathogenesis of AD. Therefore, an imaging technique that can visualize redox status of the skin could be a useful tool to monitor disease activity and therapeutic response in AD skin lesions, leading to establishment of a novel strategy in the treatment of AD from the view point of altered redox status of the skin.

In vivo dynamic nuclear polarization magnetic resonance imaging (DNP-MRI) is a method of free radical imaging in the living body. The enhancement of MR image intensity (DNP effect) derived from free radicals can be obtained by electron paramagnetic resonance (EPR) irradiation of free radical molecules [10–12]. The nitroxyl radical is nontoxic and has a stable free radical, therefore, it sensitively reacts with redox molecules within a tissue and could be widely used as a redox probe *in vivo* [13–21]. Nitroxyl radicals also have been utilized for monitoring of skin redox satus [22–24]. After redox reaction of the nitroxyl radical in tissue, it loses its free radical and is mainly converted to hydroxylamine (reduced form) resulting in decreased enhancement of image intensity in DNP-MRI.

Abbreviations: AD, atopic dermatitis; Th, T helper; ROS, reactive oxygen species; DNP, dynamic nuclear polarization; MRI, magnetic resonance imaging; EPR, electron paramagnetic resonance; SDS, sodium dodecyl sulfate; HE, hematoxylin and eosin; TB, toluidine blue

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In this study, we have for the first time established the *in vivo* imaging of redox status of lesional skin in NC/Nga mice, the animal model of AD. This model is generated by repeated topical application of mite antigen, and shows similar disease characteristics to human AD [25–27]. We have succeeded in the visualization of redox alterations in the AD skin lesions of these mice. Non-invasive monitoring of dermal tissue redox status by *in vivo* DNP-MRI could be of great value in understanding the progression of AD.

2. Materials and methods

2.1. Animals

NC/Nga Tnd mice (female mice; 4 weeks of age) were purchased from Charles River Laboratories, Inc. (Yokohama, Japan) and were housed under conventional conditions (temperature 22 ± 2 °C; humidity 50–70%; light/dark 12/12 h) with free access to water and food (MF diet, Oriental Yeast Co., Tokyo, Japan). These mice were acclimatized to their environment for 3 weeks and mice were used at 7–11 weeks old. All animal experiments were approved by the Animal Ethics Committee, Kyushu University, and were conducted in accordance with the Guidelines for Animal Experiments of Kyushu University.

2.2. Atopic dermatitis mouse model

Seven-week-old mice were randomly divided into two experimental groups; one group (n = 13) received no treatment (control mice) and the other (n = 13) received treatment for induction of AD-like lesions. On day 0, fur from the neck over the head of the mice was clipped using an electric clipper and was further depilated by hair removal cream. Following this, antigen stimulation by topical application of 100 mg of Biostir AD ointment (ointment of components derived from *Dermatophagoides farinae*; Biostir Inc., Kobe, Japan) was performed. On day 3, the stratum corneum was destructed by treatment with 100 μl of 4% SDS solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Following this, antigen stimulation by application of Biostir AD was performed. These processes were repeated twice a week and clinical scores of individual mice graded from 0 to 3 were evaluated by visual inspection, including scale, eschar, thickened skin, eruption and edema, and lesion of the ear. After four rounds of stimulation (2 weeks), the acute stage mouse model with AD-like lesions was established and designated acute-AD mice. The late stage mouse model, designated chronic-AD mice, required a total of eight rounds of stimulation (4 weeks).

2.3. Redox imaging of skin tissue with in vivo DNP-MRI

In vivo redox imaging was performed with an *in vivo* DNP-MRI system, constructed in our lab using the external magnet of a commercial EPR spectrometer (JES-ES20, JEOL Ltd., Tokyo, Japan) [21] (Fig. 1A). The external magnetic field B_0 for EPR irradiation and MRI was fixed at 20 mT, and the radiofrequency of the EPR irradiation and MRI were 527.5 MHz and 793 kHz, respectively. A rectangular one-turn curved surface coil (longitudinal direction 16 mm, lateral direction 25 mm) was used for EPR irradiation, being a high-sensitivity local detector (Fig. 1B). The scanning conditions for the *in vivo* DNP-MRI experiment were as follows: power of EPR irradiation, 5 W; flip angle, 90°; repetition time (T_R) × echo time (T_E) × EPR irradiation time (T_{EPR}), 1000 × 40 × 500 ms; coronal section; number of averages, 1; slice thickness, 100 mm; phase-encoding steps, 32; field of view (FOV), 32 × 32 mm; and matrix size, 64 × 64 after reconstruction. The surface coil was set on a special stage with a hole for probe administration. Mice were anesthetized with 2% isoflurane and placed on the stage in the supine position. Mouse body temperature during preparation was maintained on a heat insulation mat. Pharmacokinetic DNP-MR images were obtained at 2, 5, 8, 11, 14, 17, 20 min after 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (tempol) isotonic solution (2.5 mM, 100 μl; Sigma-Aldrich Chemical Co., Milwaukee, WI) was subcutaneously administered to the head. (acute-AD mice n = 5, control mice n = 5; chronic-AD mice n = 5, control mice n = 5). Then MR images were obtained without EPR irradiation. The image enhancement decay rate of each mouse was obtained from the slope of the average image intensity in the region of interest (ROI) equivalent to the enhancement by tempol using ImageJ software. A redox map was calculated from the slope of the image intensity of each pixel in seven pharmacokinetic DNP images using a custom Excel macro program.

2.4. Subcutaneous MR imaging of the mouse head

As well as the *in vivo* DNP-MR imaging, mice were anesthetized with 2% isoflurane and placed on the stage in the supine position so that the head was adjacent to the hole in the stage. Magnetic resonance images (sagittal plane) of chronic-AD mice (n = 3) and control mice (n = 3) by 1.5 T animal MRI were obtained before and after (2 min and 20 min) subcutaneous injection of tempol isotonic solution (2.5 mM, 100 μl) to the head. The scanning conditions for the MRI experiment were as follows: flip angle, 90°; repetition time (T_R) × echo time (T_E), 500 × 10 ms; sagittal section; number of accumulation, 2; slice thickness, 1 mm; phase-encoding steps, 130; sampling number,196; FOV, 60 × 60 mm; and matrix size, 256 × 256 after reconstruction. The area enhanced by administration of tempol solution was measured using ImageJ software.

2.5. Assessment of absorption of redox probe into the blood

Chronic-AD mice (n = 3) and control animals (n = 3) were subcutaneously administered tempol isotonic solution (2.5 mM, 100 μl) to the head. A blood sample was collected at 20 min after injection, then tempol radical concentration in the blood was measured by X-band EPR. The total tempol radical concentration (sum of oxidized and reduced forms) was measured after reoxidation of tempol by potassium ferricyanide (2 mM). Measurement parameters of the X-band EPR were as follows: microwave-frequency, 1 GHz; microwave-power, 1 mW; center of field, 37.8 mT; modulation width, 0.1 mT; sweep time, 10 s; sweep width, 1 mT and time constant, 0.03 s.

2.6. Histological evaluation

After the *in vivo* DNP-MRI experiments, the skin tissue of the head was removed and embedded in Surgipath FSC22 frozen section media (Leica Biosystems, Wetzlar, Germany) and stored at −80 °C (acute-AD mice n = 5, control mice n = 5; chronic-AD mice n = 5, control mice n = 5). Fresh frozen sections of 5-μm thickness were stained with HE or TB for confirmation of mast cells. Mast cells were counted under microscopy at a magnification of × 400 and were expressed as the total number of cells in five fields [27].

2.7. Serum IgE evaluation

After the *in vivo* DNP-MRI experiments, blood samples were collected from the caudal vena cava (acute-AD mice n = 5, control mice n = 5; chronic-AD mice n = 5, control mice n = 5). Serum separation was performed by centrifugation at 900g for 15 min at 4 °C after standing overnight. Serum samples were then stored at −80 °C. These samples were used for measurement of serum IgE by enzyme-linked immunosorbent assay using a mouse IgE EIA kit (Cosmo Bio Co, Ltd, Tokyo, Japan).

2.8. Statistical analysis

Unpaired Student's *t*-test or one-way analysis of variance was used to assess the results. A P value of < 0.05 was assumed to indicate a