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Mold elicits atopic dermatitis by reactive oxygen species: Epidemiology and mechanism studies

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ABSTRACT

Mold has been implicated in the development of atopic dermatitis (AD); however, the underlying mechanisms remain unknown. The aim of the study was to investigate the effects of mold exposure in early life through epidemiologic and mechanistic studies *in vivo* and *in vitro*.
Exposure to visible mold inside the home during the first year of life was associated with an increased risk for current AD by two population-based cross-sectional human studies. Children with the AG + GG genotype of *GSTP1* showed increased risk for current AD when exposed to mold. In the mouse model, treatment with patulin induced and aggravated clinically significant AD and Th2-related inflammation of the affected mouse skin. Additionally, reactive oxygen species (ROS) were released in the mouse skin as well by human keratinocytes. In conclusions, mold exposure increases the risk for AD related to ROS generation mediated by Th2-promoting inflammatory cytokines.

1. Introduction

A wide range of mold exists in the human body, and exposure to airborne spores remains fairly constant throughout the year. Recently, exposure to certain species of mold and their metabolites has caused adverse health effects, with such effects increasing worldwide due to global warming and an increasingly wetter climate [1,2]. Although several studies have reported that exposure to mold in early life is a significant risk factor for the development of allergic diseases [3,4], including atopic dermatitis (AD) in children [5–8], such findings require validation because the health effects may differ due to age at diagnosis of allergic diseases, timing of exposure to mold, and ethnicity.

AD is a chronic inflammatory skin disease caused by genetic susceptibility as well as environmental factors [9,10]. Environmental factors, particularly during critical periods, have been shown to influence the susceptibility to allergic diseases in infants and children [9]. The health effect of mold is associated with certain components of mold or metabolites such as mycotoxins, which impair the development of the immune system and generate reactive oxygen species (ROS) [11]. Genetic polymorphisms of ROS-related genes such as glutathione-S-transferase P1 (*GSTP1*) affect the catalytic activity of the ROS-generating enzyme [12], which may influence the development of allergic diseases in response to mold exposure.

Although there are studies on the association between mold exposure and generation of ROS and between generation of ROS and AD, there have been no studies on the development of AD in exposure to mold *via* ROS generation. In this study, we evaluated the association between exposure to visible mold during early infancy and in the 12 months prior to study enrollment and the development of AD in Korean children from two different epidemiologic studies. In addition, we evaluated the association between mold exposure and polymorphisms of *GSTP1* (rs1695) on the development of AD. To elucidate the plausible ROS mediated mechanisms of the development of AD in exposure to mold on the basis of our epidemiologic studies, we investigated the effects of mycotoxin in a mouse model and human primary keratinocytes (KCs).

2. Materials and methods

2.1. Study population

To evaluate the association between mold exposure and the development of AD, 2931 children between 3 and 18 years of age from the

Abbreviations: AD, atopic dermatitis; DCFH-DA, dichlorodihydrofluorescin diacetate; DMEM, Dulbecco's Modification of Eagle's Medium; DNPH, dinitrophenylhydrazine; FACS, flow cytometry; $GSTP_1$, glutathione-S-transferase P_1; IHC, immunohistochemistry; IL-4, interleukin-4; IL-5, interleukin-5; IFN-γ, interferon gamma; KCs, keratinocytes; NAC, N-acetyl cysteine; OVA, ovalbumin; ROS, reactive oxygen species; TEWL, transepidermal water loss; TSLP, thymic stromal lymphopoietin.

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Study for Standardization of Allergic Diseases [13] and 11,340 elementary and middle school students from the Study for Atopy-Free Seoul in Korea [7] were included. The two different epidemiologic studies are general population based, cross-sectional cohorts from the metropolitan and Seoul, Korea [14]. The characteristics of each study population are shown in E-Table 1. The studies were approved by the Institutional Review Board of Asan Medical Center, Ulsan University, Seoul, Korea.

2.2. Questionnaire

The questionnaire from the International Study of Asthma and Allergies in Childhood (ISAAC) was used [15]. Parents or guardians of the participants completed the questionnaire [7], which included questions on the general characteristics of the child and exposure to visible mold during the first year of life and in the 12 months prior to enrollment. Current AD was defined as physician-diagnosed AD at any point in their lifetime and the presence of eczema in the 12 months prior to study enrollment.

2.3. Genotyping

Genotyping was performed in a randomly selected group of subjects from each epidemiologic study cohort, who agreed to blood sampling for genotyping (genotyping rate: 2474/3229, 76.6% from the Study of Atopy-Free Seoul project and 667/933, 71.5% from the standardization study): 667 children from the Study for Standardization in Allergic Diseases and 1081 children from the Study for Atopy-Free Seoul. Genomic DNA from peripheral blood mononuclear cells obtained by using the Gentra® Puregene® Blood kit (QIAGEN, Maryland, USA) was screened for single nucleotide polymorphisms (SNPs) of glutathione-S-transferase P1 (*GSTP1*) (rs1695) using TaqMan assays. Assay ID number for *GSTP1* (rs1695) SNP is C_3237198_20.

2.4. Animals

Female hairless mice (SKH-1/Hr; 4 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and housed under controlled humidity (40%) and temperature (22 ± 2 °C). All mice (n = 5 per group) were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Asan Medical Center and Ulsan University College of Medicine.

2.5. Murine model of atopic dermatitis (AD)-like inflammation

Our mouse model of AD-like inflammation was used as previously described [16]. Briefly, a patch containing 100 μg ovalbumin (OVA) (grade V; Sigma, St Louis, MO) in 100 μL of normal saline was placed on the dorsum of each mouse, left there for 1 week, and then removed. After 2 weeks without the patch, another OVA-containing patch was placed on the same site of the dorsum and left there for 1 week. This procedure was repeated three times.

This protocol was also performed using 100 μg patulin in 100 μL of normal saline instead of OVA. In addition, to assess the effect of patulin on the local skin responses to OVA, 100 μg patulin (Sigma-Aldrich) in 100 μL of normal saline was added to the OVA-containing patch. As a negative control, a patch containing 100 μL of normal saline was applied.

2.6. Clinical lesion scores

Dorsum lesions were scored for erythema, scaling, and excoriation after each sensitization using a 0–3 scoring system, where 0 = no lesion, 1 = mild lesion, 2 = moderate lesion, and 3 = severe lesion. The same investigator performed all scoring evaluations throughout the study [1].

2.7. Assessment of epidermal permeability barrier function

To determine whether the epidermal permeability barrier function was altered in OVA-induced AD, transepidermal water loss (TEWL) levels of the dorsal skin were measured by using a Vapometer®SWL-3 (Delfin Technologies Ltd., Kuopio, Finland). The TEWL level was measured at baseline (before the first patch was put on) at a location that was to be covered by the patch and then again at the same site immediately after each of the three patches was removed.

2.8. Quantization of serum immunoglobulin levels

Sera were obtained from blood taken from mice on the final day of the treatment schedule. IgE-specific enzyme linked immunosorbent assay (ELISA) was used to quantify the total IgE levels in the serum. For this, matching antibody pairs (eBioscience) were used according to the manufacturer's instructions.

2.9. Histology and immunohistochemistry (IHC)

The dorsal skin that had been under the patch in the experimental mice was removed on the final day of the schedule, fixed in 10% phosphate-buffered formalin, and embedded in paraffin. Serial paraffin sections (4.5 mm thick) were stained with hematoxylin and eosin (H&E) to evaluate the degree of edema.

For IHC, the dorsal skin was processed as described previously [1]. The primary antibodies used for staining and their respective dilution rates were as follows: goat-polyclonal anti-mouse interleukin (IL)-4 antibody (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA); rabbit-polyclonal anti-mouse thymic stromal lymphopoietin (TSLP) (1:500 dilution; Abcam); rat-polyclonal anti-mouse interferon (IFN)-γ (1:1000 dilution; Abcam); and rabbit-polyclonal anti-mouse filaggrin antibody (1:1000 dilution; Covance Research, Seoul, Korea).

2.10. Determination of mast cell counts in dermal samples

Mast cells were counted using Image analysis software (ImageJ 1.47v; NIH, Bethesda, USA) at 400× magnification (high-power field). Five random fields were selected from each slide. Digital images were acquired using a microscope and digital image transfer software (BX41; Olympus, Melville, NY).

2.11. Oxidative stress staining in mouse skin

Protein oxidation was analyzed using an OxyIHC oxidative stress-detection kit (Millipore, Billerica, MA, USA) according to the manufacturer's directions. Protein carbonyl groups generated by oxidative stress were visualized using immunolabeling after reaction with 2,4-dinitrophenylhydrazine (DNPH).

2.12. Real-time RT-PCR

To measure the expression levels of IL-4, TSLP, and IFN-γ and metabolic isoenzyme during reactive oxygen species (ROS) generation, glutathione *S*-transferases (GSTs)-related genes $GSTP_1$ and $GSTM_1$, and the skin barrier protein filaggrin in murine skin, RNA from the dorsal skin that was removed from the experimental mice was extracted using RNeasy (Qiagen, Valencia, California). Real-time PCR was performed using the TaqMan method on an ABI 7500 system (Applied Biosystems, Piscataway, NJ). Signals were normalized using GAPDH.

2.13. Culture of human KCs

Primary human keratinocytes (KCs) (Cascade Biologics, Portland, OR) were grown in serum-free EpiLife cell culture medium (Invitrogen, Carlsbad, CA, USA) containing 0.06 mmol L^{-1} calcium chloride, 1%