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Chemical allergens stimulate human epidermal keratinocytes to produce lymphangiogenic vascular endothelial growth factor

Ok-Nam Bae [a], Seyeon Ahn [b], Sun Hee Jin [b], Soo Hyun Hong [b], Jinyoung Lee [b], Eun-Sun Kim [a], Tae Cheon Jeong [c], Young-Jin Chun [d], Ai-Young Lee [e,*], Minsoo Noh [b,**]

[a] College of Pharmacy, Institute of Pharmaceutical Science and Technology, Hanyang University, Ansan 426-791, Republic of Korea
[b] College of Pharmacy, Natural Products Research Institute, Seoul National University, Seoul 151-742, Republic of Korea
[c] College of Pharmacy, Yeungnam University, Gyeongsan 712-749, Republic of Korea
[d] College of Pharmacy, Chung-Ang University, Seoul 156-756, Republic of Korea
[e] Department of Dermatology, Dongguk University Ilsan Hospital, Goyang 410-773, Republic of Korea

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ABSTRACT

Allergic contact dermatitis (ACD) is a cell-mediated immune response that involves skin sensitization in response to contact with various allergens. Angiogenesis and lymphangiogenesis both play roles in the allergic sensitization process. Epidermal keratinocytes can produce vascular endothelial growth factor (VEGF) in response to UV irradiation and during wound healing. However, the effect of haptenic chemical allergens on the VEGF production of human keratinocytes, which is the primary contact site of toxic allergens, has not been thoroughly researched. We systematically investigated whether immune-regulatory cytokines and chemical allergens would lead to the production of VEGF in normal human keratinocytes (NHKs) in culture. VEGF production significantly increased when NHKs were treated with IFNγ, IL-1α, IL-4, IL-6, IL-17A, IL-22 or TNFα. Among the human sensitizers listed in the OECD Test Guideline (TG) 429, we found that CMI/MI, DNCB, 4-phenylenediamine, cobalt chloride, 2-mercaptobenzothiazole, citral, HCA, cinnamic alcohol, imidazolidinyl urea and nickel chloride all significantly upregulated VEGF production in NHKs. In addition, common human haptenic allergens such as avobenzone, formaldehyde and urushiol, also induced the keratinocyte-derived VEGF production. VEGF upregulation by pro-inflammatory stimuli, IFNγ, DNCB or formaldehyde is preceded by the production of IL-8, an acute inflammatory phase cytokine. Lymphangiogenic *VEGF-C* gene transcription was significantly increased when NHKs were treated with formaldehyde, DNCB or urushiol, while transcription of *VEGF-A* and *VEGF-B* did not change. Therefore, the chemical allergen-induced VEGF upregulation is mainly due to the increase in lymphangiogenic *VEGF-C* transcription in NHKs. These results suggest that keratinocyte-derived VEGF may regulate the lymphangiogenic process during the skin sensitization process of ACD.

Introduction

Allergic contact dermatitis (ACD) is a common immunotoxicity of human skin in response to various contact allergens. Upon first exposure, contact allergens sensitize the cutaneous immune system in human skin. In subsequent exposures, allergens elicit the cell-mediated immune reactions to trigger ACD (Kaplan et al., 2012; McFadden et al., 2013; Scott et al., 2002). During the elicitation phase, epidermal Langerhans cells and/or other dendritic cells present contact allergens to T cells in local lymph nodes. These hapten-presenting cells migrate from the epidermis to local lymph nodes via the cutaneous lymphatic system (Kimber et al., 2012).

Epidermal keratinocytes are major cellular components of human epidermal tissue and play a major role in the generation of the epidermal barrier structure. In addition, epidermal keratinocytes, a primary target of xenobiotic-associated dermal toxicity, can regulate cutaneous immunity by producing pro-inflammatory autacoids and immunoregulatory cytokines in response to various environmental toxic stressors such as chemical irritants, microbial products and ultraviolet (UV) irradiation. Due to the crosstalk between epidermal keratinocytes and the local cutaneous immune system via immunoregulatory cytokines, chemical allergen-induced cytokine production profiles in keratinocytes have been evaluated as a novel in vitro skin irritation and/or sensitization assay (Coquette et al., 2003; Son et al., 2013). In this regard, the identification of keratinocyte-derived cytokines is important in order to discover novel safety testing methods that predict the potential of new chemicals to induce ACD. Keratinocyte-derived cytokines

* Correspondence to: A.-Y. Lee, Department of Dermatology, Dongguk University Ilsan Hospital, Graduate School of Medicine, 814 Siksa-dong, Ilsandong-gu, Goyang-si, Gyeonggi-do 410-773, Republic of Korea. Fax: +82 31 961 7695.
** Correspondence to: M. Noh, College of Pharmacy, Seoul National University, Gwanak-gu, Seoul, 151-742, Republic of Korea. Fax: +82 2 880 2482.
E-mail addresses: leeay@duih.org (A.-Y. Lee), minsoo@alum.mit.edu (M. Noh).

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and chemokines, such as interleukin (IL)-1α, IL-6, IL-8, chemokine (C–C motif) ligand 2 (CCL-2, also known as MCP-1), granulocyte macrophage-colony stimulating factor (GM-CSF) and tumor necrosis factor α (TNFα), can regulate the cell-mediated immune responses to contact allergens by activating major immune cells such as lymphocytes and lymphatic endothelial cells (Kaplan et al., 2012; Pasparakis et al., 2014). Therefore, the cytokine network and the role of keratinocyte-derived epidermal cytokines are important in the understanding of the pathogenesis of ACD in skin.

The link between angiogenesis and inflammation has been suggested in chronic skin inflammation pathologies including atopic dermatitis (Huggenberger and Detmar, 2011; Thairu et al., 2011; Zhang et al., 2006). Increased angiogenesis and lymphatic endothelial remodeling are important features of chronic inflammation. Vascular endothelial growth factor (VEGF) is a major cytokine in the regulation of angiogenesis and lymphangiogenesis (Ferrara et al., 2003). VEGF is composed of four subtypes, VEGF-A, VEGF-B, VEGF-C and VEGF-D (Ruiz de Almodovar et al., 2009). VEGF-A increases the mitosis and migration of vascular endothelial cells (Pober and Sessa, 2007). VEGF-B plays a role in embryonic angiogenesis. Both VEGF-A and -B activate vascular endothelial cells to increase local blood flow, edema formation and interactions with leukocytes. While VEGF-A and -B are responsible for endothelial activation and angiogenesis, VEGF-C and -D are associated with lymphatic endothelial remodeling or lymphangiogenesis (Ferrara et al., 2003; Karkkainen and Petrova, 2000). Various pro-inflammatory stimuli, such as *Staphylococcus aureus,* peptidoglycans and UV irradiation, all promote VEGF production in murine and human keratinocytes (Kakurai et al., 2009; Kosmadaki et al., 2003; Ruiz-Gonzalez et al., 2009). The increased expression of VEGF was reported in skin lesions under chronic inflammatory dermatological conditions like psoriasis and atopic dermatitis (Detmar et al., 1994; Elias et al., 2008; Zhang et al., 2006). The importance of VEGF in chronic skin diseases has been suggested by a study reporting that systemic antagonism against VEGF in a mouse model of psoriasis significantly attenuated pathophysiologic outcomes (Schonthaler et al., 2009). Interestingly, the pathogenic role of VEGF in the respiratory allergic conditions like allergic rhinitis and allergic asthma has been reported (Choi et al., 2009; Yuksel et al., 2007), suggesting the association of epidermal VEGF with the pathogenesis of ACD. In addition, it is still unclear whether ACD-inducing haptenic chemicals directly affect cutaneous angiogenic or lymphangiogenic processes by inducing VEGF production in human epidermal keratinocytes.

In the search for the possible contribution of keratinocyte-derived VEGF to the cytokine network in cutaneous immunity, we first evaluated whether pro-inflammatory cytokines can affect human epidermal keratinocytes to produce VEGF. In addition, the systematic analysis for the chemical allergens listed in the OECD TG429 was performed for the VEGF expression in normal human keratinocytes (NHKs). IL-8 production in NHKs was measured in parallel with VEGF because IL-8 is known to be induced by various contact allergens. We found that VEGF was upregulated in NHKs in response to chemical allergens, as was IL-8, although the response profile to various chemical allergens was different between VEGF and IL-8.

Materials and methods

Cell culture and treatment with pro-inflammatory cytokines and contact allergens. Primary NHKs from neonatal foreskin were purchased from Lonza (Basel, Switzerland) and cultured as previously reported (Choi et al., 2010). NHK cells were grown to 100% confluence, and then cell culture medium was exchanged for keratinocyte basal medium (KBM, Lonza) supplemented with hEGF (KGM-2 BulletKit, Lonza) for 24 h. NHK cells, conditioned with the KBM medium with hEGF, were stimulated with recombinant human interleukin (IL)-1α (R&D systems, Minneapolis, MN, USA), tumor necrosis factor α (TNFα, R&D systems), IL-4 (R&D systems), IL-6 (R&D systems), IL-17A (Sigma Aldrich, St. Louis, MO, USA), IL-18 (R&D systems), IL-22 (R&D systems), interferon γ (IFNγ, R&D systems), avobenzone (Sigma Aldrich), formaldehyde (Sigma Aldrich), urushiol (Sigma Aldrich) or the reference chemicals for human sensitizers in OECD TG429. All chemicals listed in OECD TG429 were purchased from Sigma Aldrich, except 5-chloro-2-methyl-4-isothiazolin-3-one/2-methyl-4-isothiazolin-3-one (CMI/MI). CMI/MI was produced in a ratio of 3 to 1 by mixing CMI (Sigma-Aldrich) and MI (Sigma-Aldrich), respectively. Cell culture supernatants were harvested for measurement of IL-8 or VEGF concentrations after 24 h post-treatment. For the measurement of intracellular mRNA levels, cells were harvested for total RNA isolation 24 h after treatment.

Cell viability tests. Viability of NHK cells was evaluated using the WST-1 assay according to the manufacturer's instructions (Roche Molecular Biochemical, Indianapolis, IN, USA). The cell viability detecting reagent 4-3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate (WST-1; 10 μM pure solution) was applied to NHKs in culture, and cells were incubated for 2 h. The absorbance of the samples at 450 nm (A450) was determined using an enzyme-linked immunosorbent assay (ELISA) reader.

Determination of IL-8 and VEGF. For quantitative determination of IL-8 and VEGF in supernatants, IL-8 and VEGF DuoSet immunoassay kits were used (R&D Systems, Minneapolis, MN, USA). The chemical-treated NHK-conditioned media was centrifuged for 5 min at 1000 ×*g* and the supernatants were then diluted for use in the quantification reaction. Cytokine concentrations were determined according to the manufacturer's instructions.

RNA extraction and quantitative real time RT-PCR (Q-RT-PCR). Total RNA was obtained using TRIzol™ (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The concentration of RNA was determined spectrophotometrically, and the integrity of the RNA was assessed using a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Two micrograms of RNA was reverse-transcribed into cDNA using SuperScriptIII reverse transcriptase (Invitrogen). TaqMan Q-RT-PCR primer sets (Applied Biosystems, Foster City, CA, USA) were used to determine the transcription levels of VEGF-A (Hs00900055_m1, Applied Biosystems), VEGF-B (Hs00173634_m1, Applied Biosystems), VEGF-C (Hs00153458_m1, Applied Biosystems) and VEGF-D (Hs01128657_m1, Applied Biosystems). Human GAPDH (4333764F, Applied Biosystems) was also amplified to normalize variations in cDNA levels across different samples.

Statistical analyses. Experimental values are expressed as the mean ± SD and statistical analysis was performed using one-way ANOVA followed by Bonferroni's post-test. All statistical analyses were performed with MINITAB software (Minitab Inc. State College, PA, USA). Experiments were repeated independently three times unless otherwise stated.

Results

NHKs produce VEGF and IL-8 in response to pro-inflammatory cytokines

First, to investigate the association of keratinocyte-derived VEGF with the cytokine network in the regulation of cutaneous immunity, we treated NHKs with major pro-inflammatory cytokines and quantified the VEGF proteins that accumulated in cell culture supernatants. The cytokine concentration to elicit the response of NHKs and the ELISA measurement of VEGF after 24 h of the cytokine treatment were determined from our previous studies (Jin et al., 2014; Noh et al., 2010). Treatment with IL-1α and TNFα cytokines, which are known to be important in innate immunity, resulted in increased VEGF production by NHKs (Figs. 1A and B). IL-6, a cytokine that can affect both