

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



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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 IFN γ , IL-1 α , IL-4, IL-6, IL-17A, IL-22 or TNF α . 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, IFN γ , 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.

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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

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and chemokines, such as interleukin (IL)-1 α , 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 (TNF α), 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)-1 α (R&D systems, Minneapolis, MN, USA), tumor necrosis factor α (TNF α , 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 (IFN γ , 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 μ M 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 $\times$ 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 $\pm$ 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-1 α and TNF α 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

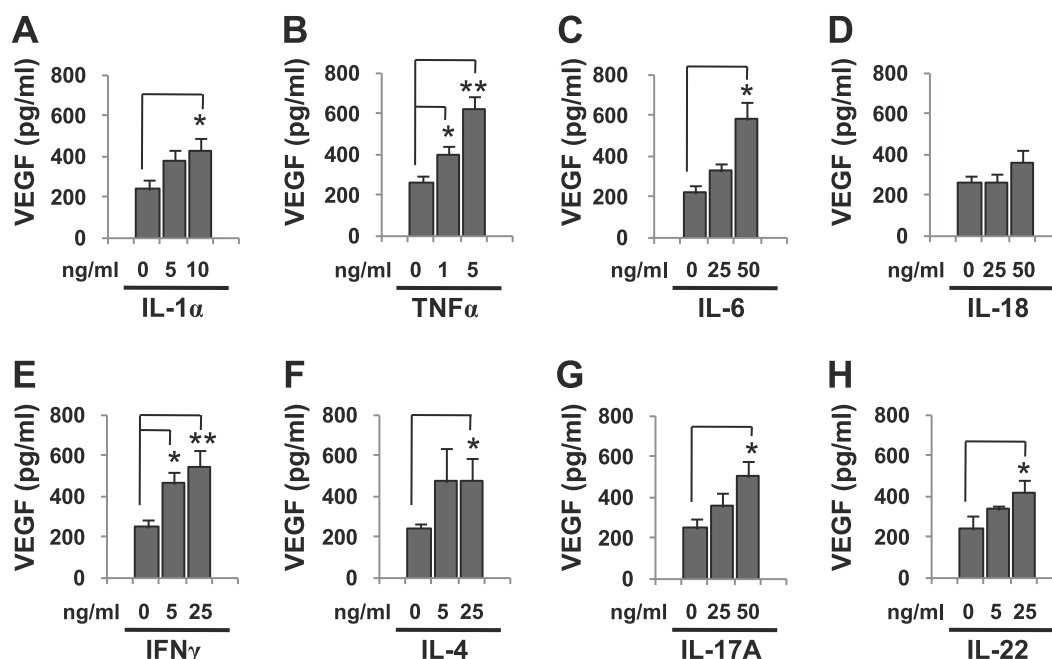


Fig. 1. Effects of pro-inflammatory cytokines on VEGF upregulation in NHKs. NHKs were cultured to confluence and then stimulated with pro-inflammatory cytokines including IL-1 α (A), TNF α (B), IL-6 (C), IL-18 (D), IFN γ (E), IL-4 (F), IL-17A (G) and IL-22 (H) for 24 h. VEGF concentrations in cell culture supernatants were quantitatively determined by ELISA. Values represent the mean expression $\pm$ SD of VEGF in the cell culture supernatant ($n = 3$). * $p \leq 0.05$ and ** $p \leq 0.01$.

epidermal barrier function and skin immunity (Turksen et al., 1992), also upregulated VEGF production in NHKs (Fig. 1C). Although the role of IL-18 in the skin sensitization process is well known (Antonopoulos et al., 2008), VEGF levels were unchanged in NHKs in response to IL-18 (Fig. 1D). Major T helper (Th) cell cytokines, IFN γ , IL-4, IL-17 and IL-22, play a pivotal role in chronic inflammatory skin diseases (Noh et al., 2010) and they also lead to increased VEGF production in NHKs (Figs. 1E–H). Of note, IFN γ and TNF α , two major cytokines associated with urushiol induced-ACD (Wakabayashi et al., 2005), significantly increased VEGF production in NHKs (Figs. 1B and E).

Chemical allergic sensitizers can stimulate NHKs to produce VEGF and/or IL-8

The mouse in vivo local lymph node assay (LLNA) is currently the preferred test for predicting the skin sensitization potential of novel chemicals (OECD, 2010; Ball et al., 2011). Not only does the OECD Test Guideline (TG) 429 recommend using the LLNA method to predict skin sensitization potential for novel chemicals, it also provides a reference chemical list of human skin irritants and sensitizers. We treated NHKs with the 22 reference chemicals suggested by the OECD TG429 and evaluated both cell viability and VEGF proteins that accumulated in cell culture supernatants over a 24-hour treatment period (Tables 1 and 2). In parallel, IL-8 levels were measured because IL-8 is a pivotal chemokine secreted from epidermal keratinocytes during ACD (Griffiths et al., 1991; Jia et al., 2012) and has also been suggested as a cytokine biomarker to predict sensitization potential in human keratinocyte and dendritic cell models (Hitzler et al., 2013; Son et al., 2013).

Of 16 human sensitizers on the TG429 chemical list, at non-cytotoxic concentrations, ten sensitizers, CMI/MI, DNCB, 4-phenylenediamine, cobalt chloride, 2-mercaptobenzothiazole, citral, HCA, cinnamic alcohol, imidazolidinyl urea and nickel chloride, significantly increased VEGF production in NHKs (Table 1). The VEGF production profile resulting from treatment with human allergic sensitizers from TG429 is different from that of IL-8. IL-8 production was significantly upregulated in NHKs in response to nine out of 16 sensitizers: DNCB, 4-phenylenediamine, isoeugenol, 2-mercaptobenzothiazole, citral, eugenol, phenylbenzoate,

cinnamic alcohol and methyl methacrylate (Table 1). DNCB, 4-phenylenediamine, citral, 2-mercaptobenzothiazole and cinnamic alcohol upregulated the production of both IL-8 and VEGF in NHKs (Table 1, Fig. 2). The human sensitizers, CMI/MI, cobalt chloride, HCA, imidazolidinyl urea and nickel chloride, significantly upregulated VEGF production in NHKs whereas they elicited no significant effect on IL-8 production (Table 1, Fig. 2). In contrast, isoeugenol, eugenol, phenyl benzoate and methyl methacrylate only increased IL-8 production in NHKs (Table 1, Fig. 2). Of the six chemical irritants from the OECD TG429 list tested, sodium lauryl sulfate (SLS) significantly increased both VEGF and IL-8 production in NHKs (Table 2). Chlorobenzene, another skin irritant on the TG429 list, upregulated IL-8 production in a concentration dependent manner, but did not affect VEGF production (Table 2).

In addition to the TG429 reference allergic sensitizers, other well-known chemical allergens were tested for their effects on VEGF production in NHKs (Fig. 3). Urushiol, a strong allergic sensitizer found in many plants, significantly increased both IL-8 and VEGF production in NHKs (Fig. 3A). Formaldehyde, a toxic chemical allergen included in the human patch test (Warshaw et al., 2013), also stimulated the production of both IL-8 and VEGF in NHKs (Fig. 3B). Avobenzone, which is widely used as a sunscreen ingredient and can elicit ACD (Sambandan and Ratner, 2011), also induced IL-8 and VEGF production in NHKs (Fig. 3C). Not all chemical allergens increased VEGF production in NHKs, however, 13 out of 19 chemical allergens we tested in this study significantly increased VEGF production (Table 1, Fig. 3).

VEGF upregulation in response to pro-inflammatory stimuli in NHKs is preceded by IL-8 production

The VEGF response profile of NHKs to allergic sensitizers is not correlated to that of IL-8 (Fig. 2), which indicates that there are mechanistically distinctive signaling pathways triggering the production of either VEGF or IL-8. To further investigate other differences in the production of VEGF and IL-8, a time-series production profile was examined in NHKs after treatment with the representative pro-inflammatory stimuli, IFN γ , DNCB or formaldehyde, inducing the upregulation of

Table 1

Effects of human skin allergens on VEGF and IL-8 production in NHKs.

OECD TG429 substance (CAS number)/ human sensitization vs. mouse model LLNA		0 (vehicle)	Chemical concentrations (μM)			
			0.4	2	10	50
CMI/MI (26172-55-4)/ human sensitizer (+), LLNA (+)	Cell survival (%)	100 ± 12	nd	103 ± 14	96 ± 7	19 ± 8**
	IL-8 (pg/ml)	62 ± 3	nd	72 ± 15	73 ± 11	96 ± 29
	VEGF (pg/ml)	145 ± 13	nd	172 ± 43	241 ± 30*	185 ± 27
DNCB (97-00-7)/ human sensitizer (+), LLNA (+)	Cell survival (%)	100 ± 5	117 ± 6	25 ± 13*	nd	nd
	IL-8 (pg/ml)	61 ± 7	146 ± 16*	7 ± 6**	nd	nd
	VEGF (pg/ml)	147 ± 22	296 ± 62*	37 ± 33**	nd	nd
4-Phenylenediamine (106-50-3)/ human sensitizer (+), LLNA (+)	Cell survival (%)	100 ± 9	97 ± 7	23 ± 11**	nd	nd
	IL-8 (pg/ml)	65 ± 9	129 ± 34*	24 ± 11**	nd	nd
	VEGF (pg/ml)	155 ± 20	295 ± 76*	46 ± 65**	nd	nd
Cobalt chloride (7791-13-1)/ human sensitizer (+), LLNA (+)	Cell survival (%)	100 ± 2	nd	103 ± 19	106 ± 9	37 ± 16*
	IL-8 (pg/ml)	61 ± 9	nd	77 ± 19	69 ± 18	92 ± 19
	VEGF (pg/ml)	149 ± 25	nd	160 ± 85	264 ± 39*	349 ± 90*
Isoeugenol (97-54-1)/ human sensitizer (+), LLNA (+)	Cell survival (%)	100 ± 20	103 ± 13	57 ± 5*	7 ± 6*	nd
	IL-8 (pg/ml)	63 ± 10	77 ± 28	135 ± 55*	11 ± 7**	nd
	VEGF (pg/ml)	158 ± 24	144 ± 48	225 ± 98	173 ± 90	nd
2-Mercaptobenzothiazole (149-30-4)/ human sensitizer (+), LLNA (+)	Cell survival (%)	100 ± 11	nd	94 ± 5	84 ± 15	51 ± 21
	IL-8 (pg/ml)	66 ± 6	nd	124 ± 7*	140 ± 14*	155 ± 32*
	VEGF (pg/ml)	159 ± 20	nd	283 ± 78	378 ± 86*	249 ± 97
Citral (5392-40-5)/ human sensitizer (+), LLNA (+)	Cell survival (%)	100 ± 10	nd	103 ± 6	72 ± 12	15 ± 8**
	IL-8 (pg/ml)	65 ± 14	81 ± 28	107 ± 25	90 ± 7*	nd
	VEGF (pg/ml)	159 ± 33	184 ± 34	234 ± 31	304 ± 74*	nd
HCA (101-86-0)/ human sensitizer (+), LLNA (+)	Cell survival (%)	100 ± 6	nd	92 ± 10	15 ± 8*	nd
	IL-8 (pg/ml)	61 ± 12	70 ± 17	61 ± 16	10 ± 4	nd
	VEGF (pg/ml)	154 ± 19	172 ± 25	181 ± 32	266 ± 36*	nd
Eugenol (97-53-0)/ human sensitizer (+), LLNA (+)	Cell survival (%)	100 ± 10	nd	63 ± 7*	18 ± 12*	nd
	IL-8 (pg/ml)	65 ± 21	95 ± 28	174 ± 32*	16 ± 14*	nd
	VEGF (pg/ml)	152 ± 8	168 ± 57	204 ± 34	118 ± 38	nd
Phenyl benzoate (93-99-2)/ human sensitizer (+), LLNA (+)	Cell survival (%)	100 ± 18	nd	107 ± 8	112 ± 8	101 ± 11
	IL-8 (pg/ml)	61 ± 10	nd	89 ± 18	101 ± 11*	135 ± 23*
	VEGF (pg/ml)	157 ± 13	nd	173 ± 30	155 ± 26	145 ± 38
Cinnamic alcohol (104-54-1)/ human sensitizer (+), LLNA (+)	Cell survival (%)	100 ± 1	nd	87 ± 24	4 ± 3**	nd
	IL-8 (pg/ml)	63 ± 12	131 ± 48*	138 ± 43*	92 ± 30	nd
	VEGF (pg/ml)	161 ± 35	189 ± 40	379 ± 54*	177 ± 39	nd
Imidazolidinyl urea (39236-46-9)/ human sensitizer (+), LLNA (+)	Cell survival (%)	100 ± 10	nd	99 ± 4	11 ± 9**	nd
	IL-8 (pg/ml)	67 ± 16	101 ± 47	102 ± 26	1 ± 1**	nd
	VEGF (pg/ml)	160 ± 23	205 ± 48	309 ± 86*	79 ± 40	nd
Methyl methacrylate (80-62-6)/ human sensitizer (+), LLNA (+)	Cell survival (%)	100 ± 9	nd	104 ± 6	103 ± 9	93 ± 10
	IL-8 (pg/ml)	68 ± 7	nd	69 ± 12	100 ± 17*	44 ± 14*
	VEGF (pg/ml)	145 ± 13	nd	160 ± 35	154 ± 42	171 ± 39
Isopropanol (67-63-0)/ human sensitizer (+), LLNA (-)	Cell survival (%)	100 ± 5	nd	110 ± 13	107 ± 12	102 ± 21
	IL-8 (pg/ml)	65 ± 6	nd	73 ± 17	86 ± 8	54 ± 11
	VEGF (pg/ml)	155 ± 30	nd	177 ± 41	202 ± 55	205 ± 43
Ethylene glycol dimethacrylate (97-90-5)/ human sensitizer (+), LLNA (+)	Cell survival (%)	100 ± 6	nd	104 ± 4	52 ± 20	13 ± 8**
	IL-8 (pg/ml)	57 ± 6	77 ± 14	103 ± 7	3 ± 2	nd
	VEGF (pg/ml)	134 ± 8	165 ± 52	164 ± 59	195 ± 73	nd
Nickel chloride (NiCl) (7718-54-9)/ human sensitizer (+), LLNA (-)	Cell survival (%)	100 ± 16	nd	106 ± 12	88 ± 16	30 ± 10*
	IL-8 (pg/ml)	54 ± 11	nd	64 ± 22	96 ± 37	61 ± 42
	VEGF (pg/ml)	155 ± 9	nd	173 ± 33	333 ± 65*	219 ± 39

LLNA is the mouse local lymph node assay. 'nd' denotes 'not determined', CMI/MI = 5-chloro-2-methyl-4-isothiazolin-3-one/2-methyl-4-isothiazolin-3-one, DNCB = 2,4-dinitrochlorobenzene, HCA = hexyl cinnamic alcohol.

* Denotes $p < 0.05$ ($n = 3$).

** Denotes $p < 0.01$ ($n = 3$), when compared to the values of vehicle-treated samples.

both VEGF and IL-8 (Fig. 4). In the time-course analysis, IL-8 upregulation was detected in NHKs 8 h after the treatment (Fig. 4A). However, VEGF levels were not significantly different from that in control at the 8-hour timepoint (Fig. 4B). Significant changes in both VEGF and IL-8 protein levels were detected 24 h after the treatment (Fig. 4B). At 48 h, the upregulation of both IL-8 and VEGF protein levels was still detected in the culture supernatants of NHKs. Therefore, the upregulation of IL-8 preceded the increase in VEGF production in NHKs in response to IFN γ , DNCB or formaldehyde (Fig. 4). IL-8 plays a role in the acute phase of inflammation, whereas VEGF is known to participate in the transitional or resolving phase of inflammation (Kataru et al., 2009; Ortega-Gómez et al., 2013). To understand the physiological role of VEGF upregulation in response to immune stimuli like IFN γ and chemical allergens such as DNCB and formaldehyde, it was essential to identify the VEGF subtypes involved in the overall upregulation of VEGF protein expression in NHKs.

VEGF-C gene transcription is increased in NHKs in response to allergic sensitizers

To characterize the VEGF subtypes responsible for the upregulation of VEGF protein production in NHKs after treatment with TNF α , IFN γ , IL-4, IL-17A, SLS, formaldehyde, DNCB or urushiol, the transcription of VEGF-A, VEGF-B, VEGF-C and VEGF-D mRNA was evaluated by quantitative real time RT-PCR (Q-RT-PCR) (Fig. 5). Among pro-inflammatory cytokines, TNF α increased the gene transcription of VEGF-A (Fig. 5A). IFN γ and IL-4 tended to increase mRNA levels, however, the p values were 0.064 and 0.14 in a comparison with the control, respectively. SLS also significantly increased the gene transcription of VEGF-A (Fig. 5A). IL-17A, formaldehyde, DNCB and urushiol had no effect on the gene transcription of VEGF-A. TNF α , IFN γ , IL-4, IL-17A, SLS, formaldehyde, DNCB or urushiol had no effect on the mRNA levels of VEGF-B genes in NHKs (Fig. 4B). The gene transcription of VEGF-C, however,

Table 2

Effects of human skin irritants on VEGF and IL-8 production in NHKs.

OECD TG429 substance (CAS number)/ human sensitizer vs. mouse model LLNA		0 (vehicle)	Chemical concentrations (μM)		
			2	10	50
Chlorobenzene (108-90-7)/ human sensitizer (–), skin irritant (+), LLNA (–)	Cell survival (%)	100 ± 2	107 ± 10	94 ± 18	94 ± 21
	IL-8 (pg/ml)	64 ± 18	97 ± 32	129 ± 22*	164 ± 52*
	VEGF (pg/ml)	148 ± 21	197 ± 61	177 ± 28	216 ± 62
Lactic acid (50-21-5)/ human sensitizer (–), skin irritant (+), LLNA (–)	Cell survival (%)	100 ± 8	99 ± 10	93 ± 9	15 ± 4**
	IL-8 (pg/ml)	69 ± 16	55 ± 4	75 ± 11	70 ± 6
	VEGF (pg/ml)	160 ± 31	219 ± 81	187 ± 56	211 ± 73
Methyl salicylate (119-36-8)/ human sensitizer (–), skin irritant (+), LLNA (–)	Cell survival (%)	100 ± 9	109 ± 28	105 ± 15	14 ± 4**
	IL-8 (pg/ml)	65 ± 14	61 ± 5	66 ± 23	41 ± 12
	VEGF (pg/ml)	147 ± 25	144 ± 16	209 ± 77	130 ± 39
Salicylic acid (69-72-7)/ human sensitizer (–), skin irritant (+), LLNA (–)	Cell survival (%)	100 ± 3	92 ± 12	84 ± 16	24 ± 23*
	IL-8 (pg/ml)	59 ± 12	70 ± 18	92 ± 6	139 ± 34*
	VEGF (pg/ml)	141 ± 20	133 ± 37	159 ± 45	223 ± 98
Sodium lauryl sulfate (SLS) (151-21-3)/ human sensitizer (–), skin irritant (+), LLNA (+)	Cell survival (%)	100 ± 8	100 ± 11	55 ± 16	13 ± 13
	IL-8 (pg/ml)	61 ± 6	78 ± 16	111 ± 34*	nd
	VEGF (pg/ml)	153 ± 18	234 ± 47	438 ± 95*	nd
Xylene (1330-20-7) human sensitizer (–), skin irritant (+), LLNA (+)	Cell survival (%)	100 ± 17	94 ± 5	98 ± 11	67 ± 26
	IL-8 (pg/ml)	54 ± 6	93 ± 12	110 ± 11	119 ± 10
	VEGF (pg/ml)	155 ± 10	156 ± 20	163 ± 41	193 ± 16

LLNA is the mouse local lymph node assay. 'nd' denotes 'not determined'.

* Denotes $p < 0.05$ ($n = 3$).** Denotes $p < 0.01$ ($n = 3$), when compared to the values of vehicle-treated samples.

was significantly enhanced in NHKs by treatment with TNF α , IFN γ , IL-4, IL-17A, SLS, formaldehyde, DNCB or urushiol (Fig. 4C). The mRNA signal for VEGF-D was not detected in NHKs up to the 40th PCR cycle (data not shown). TNF α and SLS affected the gene transcription of both angiogenic VEGF-A and lymphangiogenic VEGF-C. In contrast to these pro-inflammatory mediators, major chemical allergens, formaldehyde, DNCB and urushiol, only affected the VEGF-C gene transcription. These results suggest that the upregulation of VEGF in NHKs by allergic sensitizers is not due to a change in angiogenic VEGF-A/-B, but to the upregulation of lymphangiogenic VEGF-C, indicating that the upregulation of VEGF in NHKs is related to lymphangiogenic promotion of the sensitization process as it relates to ACD.

Discussion

In ACD, the epidermal keratinocytes are the primary contact site of haptenic chemical allergens and their reactive metabolites. Keratinocytes are a predominant cell component of the human epidermis and can modulate cutaneous immune responses from environmental stressors by secreting pro-inflammatory cytokines. In this study, we demonstrated that the major immune modulating cytokines, IL-1 α , TNF α , IL-6, IFN γ , IL-4, IL-17A and IL-22, stimulate NHKs to produce VEGF, which suggests that VEGF may play a pivotal role in the cytokine network to regulate epidermal immunity and to affect the pathogenesis of various dermatological conditions. In addition, we provided the

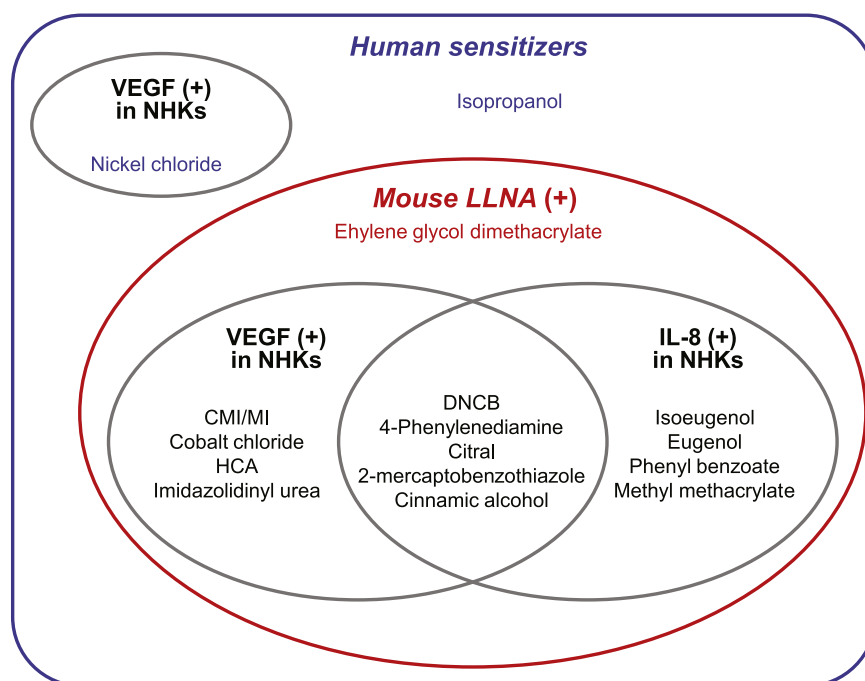


Fig. 2. The Venn diagram representing the effects of OECD TG429 chemical sensitizers on VEGF and/or IL-8 production in NHKs. The effects of 16 chemical sensitizers in Table 1 were classified in the Venn diagram according to the significant upregulation of VEGF and/or IL-8 in NHKs. A comparison is provided for the chemicals that tested positively in the in vivo LLNA test.

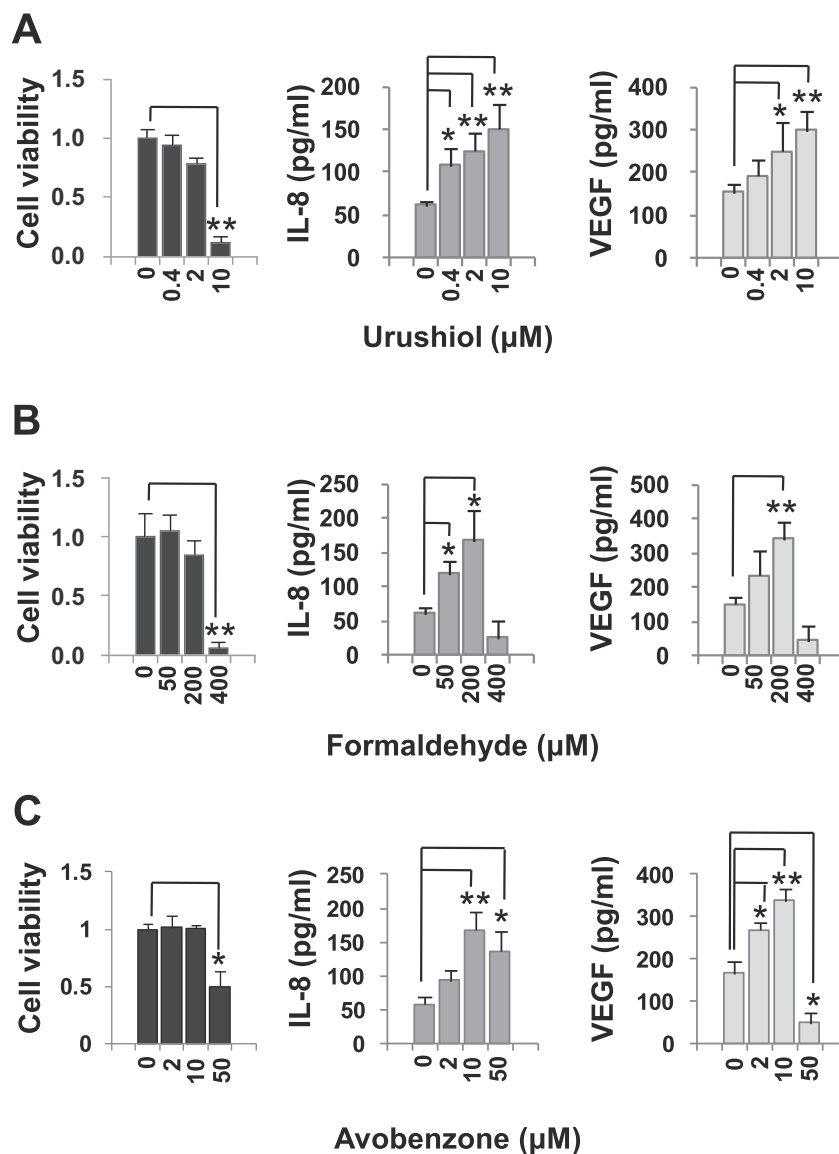


Fig. 3. Effects of formaldehyde, urushiol, and avobenzone on the VEGF and/or IL-8 increase in NHKs. NHKs were cultured to confluence and then stimulated with formaldehyde (A), urushiol (B) and avobenzone (C) for 24 h. Cell viability was determined by WST-1. The protein levels of VEGF and IL-8 in the culture supernatants of NHKs were measured by ELISA. Values represent the mean expression $\pm$ SD ($n = 3$). * $p \leq 0.05$ and ** $p \leq 0.01$.

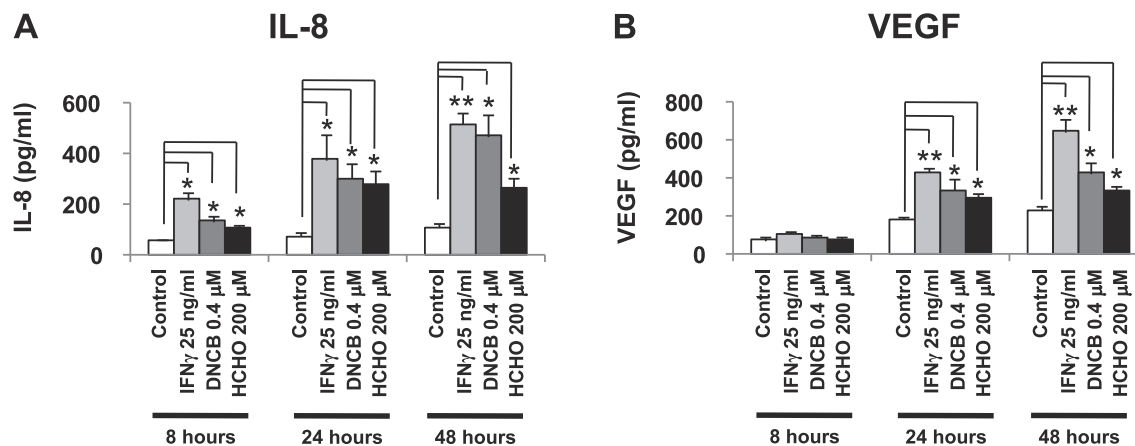


Fig. 4. The temporal expression profile of VEGF and IL-8 in NHKs. Confluent NHKs were treated with IFN γ (25 ng/ml), DNCB (0.4 μM) and formaldehyde (HCHO; 200 μM). Cell culture supernatants were harvested at 8, 24 and 48 h after treating NHKs with IFN γ , DNCB and formaldehyde. ELISA was performed for IL-8 (A) and VEGF (B). Values represent the mean expression $\pm$ SD ($n = 3$). * $p \leq 0.05$ and ** $p \leq 0.01$.

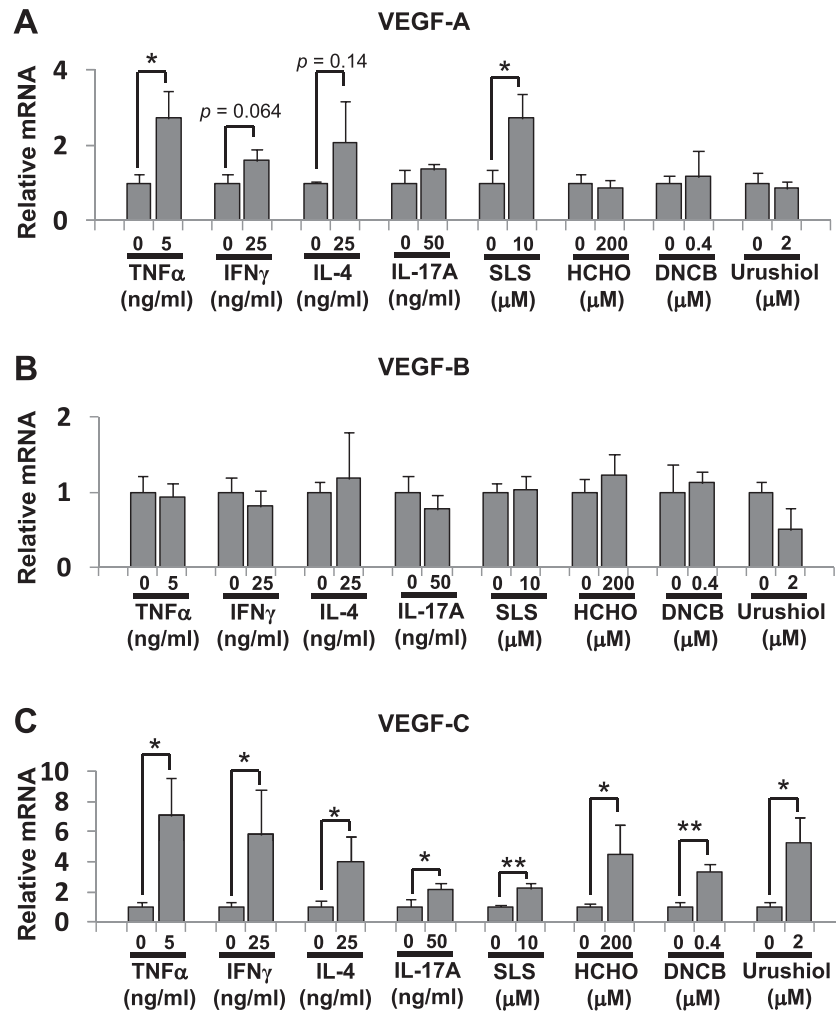


Fig. 5. The effects of VEGF-inducing stimuli on the expression of VEGF isotypes by NHK. Confluent NHKs were treated with TNF α (5 ng/ml), IFN γ (25 ng/ml), IL-4 (25 ng/ml), IL-17A (50 ng/ml), SLS (10 μ M) formaldehyde (HCHO; 200 μ M), DNCB (0.4 μ M) and urushiol (2 μ M) for 24 h. Total RNA was extracted and Q-RT-PCR was performed for VEGF-A (A), VEGF-B (B) and VEGF-C (C). Values represent the mean expression $\pm$ SD of the mRNA of the VEGF isotype genes relative to human GAPDH expression ($n = 3$). * $p \leq 0.05$ and ** $p \leq 0.01$.

evidence that most allergenic chemical sensitizers increase VEGF production by NHKs. These results suggest that VEGF produced by epidermal keratinocytes may play a role in the pathogenesis of hapten-induced ACD, or more specifically, the induction of cutaneous angiogenesis or lymphangiogenesis during the sensitization process.

Cell viability is commonly used as an indicator for the toxic potential of chemicals. However, in the case of skin sensitization, the use of pathogenic mechanism-based biomarkers, such as inflammatory cytokine mediators, may increase predictive power for determining ACD-inducing potential (OECD, 2010; Jang et al., 2014). Keratinocyte-derived cytokines such as IL-1 α , IL-8 and CCL-2 have been studied as predictors of in vitro skin irritation and/or sensitization (Coquette et al., 2003; Son et al., 2013). For the development of novel in vitro skin sensitization test methods, various biological effects of skin allergenic sensitizers have been evaluated (Gerberick et al., 2007; Natsch and Emter, 2008; Sakaguchi et al., 2010). For example, pro-inflammatory cytokines, such as IL-1 α or IL-8 have long been produced in mammalian cell culture systems and serve as potential biomarkers for new in vitro methods (Coquette et al., 2003; Natsch and Emter, 2008; Son et al., 2013). The upregulation pattern of IL-1 α and/or IL-8 by skin sensitizers and irritants has been suggested as a method for differentiating between sensitizers and irritants (Coquette et al., 2003). The combination of IL-1 α and MIP-2 (macrophage inflammatory protein-2) expression in keratinocyte cell lines can be used to measure the sensitizing potential of OECD TG429 reference compounds (Son

et al., 2013). The expression of IL-8 is suggested to be an in vitro screening method for skin sensitization potential, especially in dendritic cell-like THP-1 (Nukada et al., 2008; Takahashi et al., 2011; Hitzler et al., 2013). Despite the extensive efforts to validate sensitization biomarkers in epidermal cells such as keratinocytes and dendritic cell lines, more comprehensive and mechanism-based biomarkers must be identified with improved performance to predict skin sensitization potential. In this regard, we suggest that the co-measurements of VEGF and other cytokines expressed in the immediate, early phase of inflammation in NHKs may provide better predictive sensitivity of their allergenic potential. In this study, 14 out of the 16 human sensitizers of the OECD TG429 reference compounds significantly increased either IL-8 or VEGF in NHKs, showing 87.5% test sensitivity (Fig. 2). The mouse LLNA method also showed 87.5% test sensitivity to predict allergic sensitization potential (OECD, 2010). Ethylene glycol dimethacrylate, a human sensitizer, which tested positively using the LLNA method, did not change either VEGF or IL-8 protein levels (Table 1, Fig. 2). Interestingly, nickel chloride, a human sensitizer resulting in a negative result using the LLNA method, significantly changed VEGF protein expression in NHKs (Fig. 2). Isopropanol, classified as a human sensitizer by the OECD TG429 list, had no effect on VEGF or IL-8 production. Of the six skin irritants on the OECD TG429 list, both IL-8 and VEGF were upregulated in NHKs by SLS (Table 2). In the standpoint of the prediction of allergenic potential, the cytokine biomarkers, IL-8 and VEGF, may give rise to a false-positive result of chemical irritants like SLS. However, SLS is also

categorized as a false-positive compound in the mouse LLNA method (OECD, 2010). Therefore, the predictive potential of the co-measurement of both VEGF and IL-8 in NHKs is comparable to the mouse LLNA method (Table 1, Fig. 2). On the whole, these results suggest that the combinatorial measurements of multiple cytokine biomarkers in NHKs, such as VEGF and IL-8, can be employed as complementary methods, thereby giving rise to a novel in vitro predictive safety testing method for ACD.

The VEGF upregulation by IFN γ , DNCB and formaldehyde is preceded by the IL-8 upregulation in NHKs (Fig. 4). A temporal expression profile of immunoregulatory cytokines in epidermal keratinocytes is crucial to the orchestration of inflammatory responses to external, toxic stimuli such as chemical allergens (Kataru et al., 2009). IL-8 functions as a chemokine, which recruits polymorphonuclear leukocytes, mainly neutrophils, into inflamed tissue in the early phase of inflammation (Natsch and Emter, 2008; Son et al., 2013). In contrast, VEGF has been reported to play its critical role during the resolution phase of inflammation (Kataru et al., 2009). VEGF upregulation has been well addressed in the skin wound healing process (Brown et al., 1992). Regarding the resolution process during the wound healing-associated inflammation, the role of VEGF in the removal of activated macrophages was reported (Petreaca et al., 2008). Considering the temporal responses during inflammation, chemical allergens may induce IL-8 earlier than VEGF in NHKs, although a systematic evaluation with many other sensitizers would be required to verify that possibility.

Importantly, we found that the gene transcription of lymphangiogenic VEGF-C was upregulated by treating NHKs with allergic sensitizers formaldehyde, DNCB or urushiol, whereas the transcription of VEGF-A and VEGF-B was unaffected. In contrast, TNF α , IL-4 and a skin irritant SLS significantly increased the gene transcription of both VEGF-A and VEGF-C. Therefore, allergic sensitizers preferentially increased VEGF-C production in human epidermal keratinocytes. VEGF-C is known to bind VEGF receptor type 3, which has a unique role in lymphatic endothelial cell development (Olsson et al., 2006). Additionally, the enlargement of lymphatic vessels was reported in the skin from VEGF-C-transgenic mice (Jeltsch et al., 1997). In contrast, lymphatic hypoplasia and lymphedema were cutaneous phenotypes of the VEGF-C^{+/-} mice, indicating that cutaneous lymphangiogenesis is suppressed (Karkkainen et al., 2004). Recently, it was reported that tumor promoter-induced inflammation was suppressed in transgenic mice that overexpressed soluble VEGF-C receptors, suggesting a pivotal role of VEGF-C in cutaneous inflammation (Alitalo et al., 2013). The increase of VEGF-C in NHKs in response to chemical allergens, such as DNCB and formaldehyde, indicates a potential role for VEGF-C in the sensitization process of ACD development, or more specifically, chemical allergen-induced lymphangiogenesis or lymphatic endothelial remodeling. We hypothesize that the chemical allergen-induced upregulation of VEGF-C may promote local lymphangiogenesis during the sensitization process. The increased lymphangiogenesis may favor the migration of hapten-stimulated epidermal dendritic cells or Langerhans cells toward local lymph nodes. However, further studies will be necessary to experimentally validate this hypothesis of the association between the skin sensitizer-induced upregulation of VEGF-C and ACD-related lymphangiogenesis.

In conclusion, we demonstrated that VEGF is upregulated in NHKs in response to inflammatory cytokines and/or chemical allergens. Importantly, lymphangiogenic VEGF-C is increased in NHKs in response to treatment with chemical allergens, underlining its potential role in the sensitization or amplification phase of immune responses in human skin. This study provides the first evidence that the chemical allergen-induced VEGF upregulation in NHKs can be used as a novel biomarker to predict the skin sensitization potential of chemicals.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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