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Keratinocytic Vascular Endothelial Growth Factor as a Novel Biomarker for Pathological Skin Condition

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Abstract

Skin is an emerging target tissue in pharmaceutical and cosmetic science. Safety assessment for dermal toxicity is a critical step for development of topically applicable pharmaceutical agents and ingredients in cosmetics. Urgent needs exist to set up toxicity testing methods for dermal safety, and identification of novel biomarkers for pathological cutaneous alteration is highly required. Here we will discuss if vascular endothelial growth factor (VEGF) has a potential as a biomarker for dermal impairment. Experimental and clinical evidences for induction of keratinocytic VEGF under pathological conditions will be reviewed.

Key Words: Vascular endothelial growth factor (VEGF), Biomarker, Keratinocytic damage, Dermal toxicity

INTRODUCTION

The interest on skin has been increased both in pharmaceutical and cosmetic industries. Epidermal tissue composes of a physical barrier structure, maintaining homeostasis by preserving water and protecting inner organs against various external stresses including chemicals, microbial products, and UV irradiation. Keratinocytes are the main components of epidermis, and play active roles in skin function. In many skin problems such as allergic contact dermatitis, atopic dermatitis, psoriasis and photo-toxicity, serious damage and functional impairment such as epidermal barrier dysfunction, impaired differentiation/proliferation and dysregulated intercellular communication in keratinocytes are observed (Kubo *et al*., 2012; Hänel *et al*., 2013). There have been intensive efforts to elucidate the pathogenic alteration of keratinocytes and identify new biomarkers for keratinocytic damage during skin diseases (Enerbäck, 2011; Bernard *et al*., 2012). Here we will discuss the potential of vascular endothelial growth factor (VEGF) as a novel biomarker for keratinocyte damage, by examining experimental and clinical evidences for the role of VEGF in skin disorders.

BASIC CHARACTERISTICS OF VEGF AND VEGFR

VEGF, a dimeric heparin-binding glycoprotein of approximately 40 kDa in its active form, is known to be a main regulator of physiological and/or pathological angiogenesis. Since it was first described as vascular permeability factor (VPF) (Senger *et al*., 1983; Keck *et al*., 1989), several VEGF sub-family members and isoforms have been reported. Although the existence of VEGF-E and -F is newly suggested (Suto *et al*., 2005; Takahashi and Shibuya, 2005), it is generally accepted that VEGF sub-family consists of five members, VEGF-A, B, C, D and placenta growth factor (PLGF) in mammals (Olsson *et al*., 2006). Due to the alternative splicing of the original mRNA transcript, VEGF is occurring in isoforms with different biological activities. In case of VEGF-A, at least four isoforms of VEGF-A121, 165, 189 and 206 exist (Tischer *et al*., 1991; Gille *et al*., 2000). The bioactivity of VEGF sub-family members is also affected by proteolytic processing which enables specific interactions with different receptor types (Lee *et al*., 2005).

VEGF selectively binds to high affinity tyrosine kinase receptors (RTKs) that are predominantly expressed on endothelial cells (ECs) (de Vries *et al*., 1992; Olsson *et al*., 2006), resulting in receptor activation and intracellular signal transduction.

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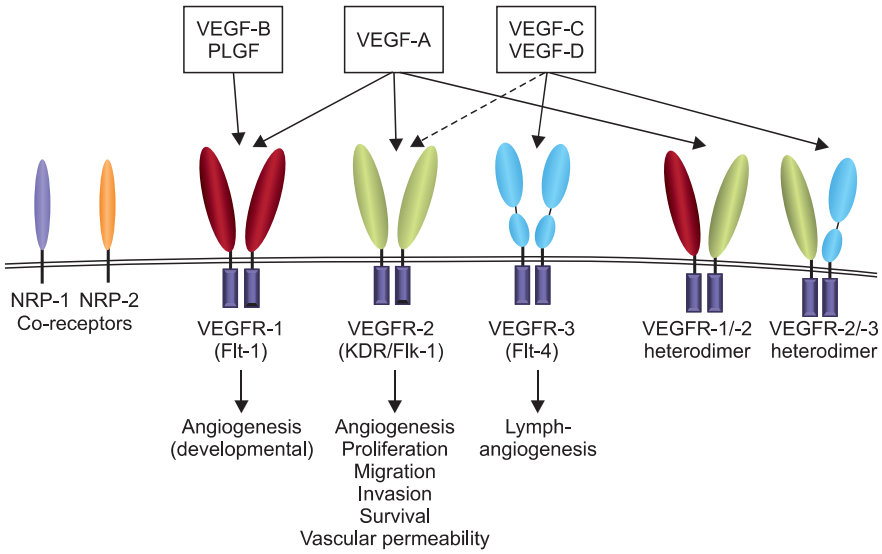

Fig. 1. Interactions between VEGF and VEGF receptors, and their biological functions. Flt, fms-like tyrosine kinase; Flk, fetal liver kinase; NRP, neuropilin; KDR, kinase insert-domain containing receptor; PLGF, placenta growth factor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

There are three types of VEGF receptors, which are VEGFR-1 (fms-like tyrosine kinase-1 or Flt-1), VEGFR-2 (kinase insert-domain containing receptor (KDR) or fetal liver kinase (Flk-1)), and VEGFR-3 (Flt-4) (Skobe *et al*., 1999; Carmeliet, 2000). VEGF receptors usually form homodimers, but heterodimeric complexes of VEGF receptor are also expressed. With different affinities and selectivities, each of VEGF sub-family members binds to distinct VEGFRs (Ruiz de Almodovar *et al*., 2009). Specific interaction between VEGF sub-family members and VEGFR is shown in Fig. 1. The neuropilin receptors (NRP-1 and 2) are known to enhance VEGF signaling by modulating VEGF-VEGFR interaction as co-receptors (Geretti *et al*., 2008).

The overall regulatory mechanism of VEGF receptor signaling is similar to typical RTK signaling of other growth factors, such as cellular signaling in cell migration, proliferation and survival (Leung *et al*., 1989; Ruiz de Almodovar *et al*., 2009). Besides these typical roles, the unique bioactivity of VEGF receptor is to transduce signals for angiogenesis and lymphangiogenesis, and to regulate permeability (Ferrara *et al*., 2003; Olsson *et al*., 2006). VEGFR-1 mainly binds to VEGF-A and B, and plays key roles in developmental/embryonic angiogenesis. The majority of angiogenic activities of VEGF, such as EC proliferation, migration, and microvascular permeability, are mediated by VEGFR-2 following binding with VEGF-A. Meantime, VEGFR-3 is predominantly found in lymphatic ECs, and promotes lymphangiogenesis by binding with VEGF-C and D (Jeltsch *et al*., 1997; Hicklin and Ellis, 2005; Olsson *et al*., 2006; Zgraggen *et al*., 2013).

VEGF SYNTHESIS IN KERATINOCYTES

Following the initial *in vivo* observation of expression of VEGF mRNA in the newly generated epithelium (Brown *et al*., 1992), the source of cutaneous VEGF was extensively studied. It is possible that VEGF during wound repair can be induced by macrophages or fibroblast (Nissen *et al*., 1998, Trompezinski *et al*., 2004), but keratinocytes are found to be one of the main sources of cutaneous VEGF. In cultured human keratinocytes, significant up-regulation of VEGF was observed after stimulation with serum, epidermal growth factor (EGF), transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α), insulin-like growth factor-2, or keratinocyte growth factor (Frank *et al*., 1995; Kim and Kim, 2005). Three major spliced forms of VEGF can be synthesized in human keratinocytes that are the secreted 121 and 165 isoforms and cell-associated 189 isoform (Ballaun *et al*., 1995). Acting as a key mediator for cutaneous angiogenesis and vascular permeability, keratinocytic VEGF is a potent and selective mitogen for dermal microvascular ECs during physiological processes such as wound repair (Wilgus *et al*., 2005) and hair growth (Yano *et al*., 2001), and pathological conditions including cutaneous inflammation, skin cancer (Brown *et al*., 1992) and psoriasis (Detmar *et al*., 1994).

SIGNALING PATHWAYS IN VEGF INDUCTION IN KERATINOCYTES

Generally, the expression of VEGF is tightly regulated at the transcriptional level and also at the post-transcriptional level (Ruiz de Almodovar *et al*., 2009). Various growth factors and cytokines are known to induce VEGF expression in keratinocytes, including interleukins (ILs), TGF-α, TGF-β, TNF-α, EGF, and platelet-derived growth factor (PDGF) (Frank *et al*., 1995; Cohen *et al*., 1996; Gille *et al*., 1997; Kozlowska *et al*., 1998; Ma *et al*., 2014). Contribution of signaling kinases including phosphatidylinositol 3-kinase (PI3K) or mitogen-activated protein kinase (MAPK; p42/p44 MAPK) to VEGF synthesis in keratinocytes are well-established (Nakai *et al*., 2009; Yu *et al*., 2010). Transcription factors of activation protein (AP)-1, AP-2, hypoxia-induced factors (HIFs), specificity protein (SP)-1 and nuclear factor κB (NFκB) are found to mediate transcriptional

Table 1. Keratinocyte derived VEGF under pathological conditions in skin

Pathological condition	Evidences for VEGF involvement	Suggested roles of VEGF	Refs
Cutaneous inflammation	Increased expression of VEGF/VEGFR in skin lesion of inflammation VEGF induction by inflammatory cytokines such as ILs and TNF-α VEGF-induced immune cell accumulation in the skin	Hyper-permeability Angiogenesis Lymphangiogenesis	Detmar *et al*., 1994; Elias *et al*., 2008; Huggenberger and Detmar, 2011; Suzuki *et al*., 2014; Zhang *et al*., 2006
Psoriasis	Increased expression of VEGF/VEGFR in skin lesion of psoriasis Increased level of systemic serum VEGF in psoriasis Aggravated psoriasis in VEGF transgenic animal Reduced psoriatic symptoms after systemic antagonism against VEGF-VEGFR Susceptibility change in psoriasis by VEGF genetic polymorphism	Epidermal hyperplasia Hyper-permeability Inflammation Enragement of lymphatic vessels	Bhushan *et al*., 1999; Canavese *et al*., 2010; Detmar *et al*., 1994, 1998; Elias *et al*., 2008; Nielsen *et al*., 2002; Rogers and D'Amato, 2006; Schonthaler *et al*., 2009; Weidemann *et al*., 2013; Xia *et al*., 2003; Young *et al*., 2006
Phototoxicity	UV-induced VEGF induction in skins and keratinocytes Increased level of VEGFR by UV irradiation Increased susceptibility to photo-damage by VEGF	Hyper-permeability Edema Erythema Epidermal hyperplasia Inflammation	Blaudschun *et al*., 2000; Brauchle *et al*., 1996; Brenneisen *et al*., 2003; Gille *et al*., 2000; Hirakawa *et al*., 2005; Longuet-Perret *et al*., 1998; Yano *et al*., 2005
Skin cancer	VEGF up-regulation in lesions of skin cancers Reduced invasion by blocking of VEGF-VEGFR Increased invasion by over-expressed VEGF	Angiogenesis Lymphangiogenesis Inflammation Invasion	Alitalo *et al*., 2013; Detmar *et al*., 1995; Gille *et al*., 2000; Hicklin and Ellis, 2005; Mantovani *et al*., 2008

regulation of VEGF in keratinocytes (Forsythe *et al*., 1996; Finkenzeller *et al*., 1997; Gille *et al*., 2000, Brenneisen *et al*., 2003; Scortegagna *et al*., 2008). Several pathological conditions such as hypoxia or UV irradiation induce up-regulation of keratinocytic VEGF (Detmar *et al*., 1997; Gille *et al*., 2000; Brenneisen *et al*., 2003; Weir *et al*., 2011), either by direct activation of transcriptional pathways or by secretion of cytokines.

UP-REGULATION OF VEGF IN SKIN DAMAGE

Although it is clear that VEGF can be expressed in keratinocytes, it is still controversial whether cutaneous VEGF is restorative or aggravative for skin damage. In normal epidermis, blood vessels are generally quiescent and angiogenesis is hardly occurring. Consistently, the level of VEGF in normal epidermis is found to be low (Weninger *et al*., 1996). However, in many skin conditions such as psoriasis, contact dermatitis, wound healing and cutaneous neoplasia that are closely associated with angiogenesis or chronic inflammation, there is prominent induction of VEGF in epidermal keratinocyte (Detmar, 1996; 2000) suggesting that increased VEGF plays key roles in these skin problems. The evidences and the suggested roles of up-regulated VEGF in abnormal skin condition are summarized in Table 1.

Cutaneous inflammation

Inflammation is basically a self-defensive innate immune response against harmful stimuli including infectious agents, physical or chemical challenges. For infiltration of inflammatory cells to inflamed tissue, change in vascular permeability is inevitable. Besides the hyper-permeability, the close association between angiogenesis and inflammation is reported in various skin diseases that require vascular remodeling (Detmar *et al*., 1994; Karkkainen and Petrova, 2000). Angiogenesis and lymphangiogenesis occur in chronic cutaneous inflammations in atopic dermatitis and psoriasis (Detmar *et al*., 1994; Zhang *et al*., 2006; Elias *et al*., 2008; Huggenberger and Detmar, 2011). Hyper-permeability and angiogenesis/lymphangiogenesis are mainly mediated by VEGF, therefore it is convincing that VEGF is up-regulated in lesions with cutaneous inflammation (Detmar *et al*., 1994; Elias *et al*., 2008; Zhang *et al*., 2006). It is also interesting that pro-inflammatory cytokines up-regulate VEGF in keratinocytes. Temporal expression profile of cytokines in epidermal keratinocytes is important in the orchestration of inflammatory responses (Kataru *et al*., 2009). IL-1, 6, 8 and TNF-α are known to be potent inducers for keratinocyte-derived VEGF-A (Detmar *et al*., 1995). VEGF-C induction associated with up-regulated VEGFR-3 and dermal lymphangiogenesis were observed in the lesion of atopic dermatitis in IL-4 transgenic mouse (Shi *et al*., 2012), supporting the role of VEGF in IL-mediated lymphangiogenesis and inflammation. The role of VEGF as a chemotactic factor in skin inflammation was also reported (Suzuki *et al*., 2014)

Psoriasis

The link between pathological skin condition and VEGF is well-established in psoriasis by clinical and experimental data (Elias *et al*., 2008; Schonthaler *et al*., 2009), and it is even suggested that systemic VEGF antagonist can be a therapeutic option for psoriasis treatment (Canavese *et al*., 2010; Weidemann *et al*., 2013). The systemic serum VEGF level (Bhushan *et al*., 1999; Nielsen *et al*., 2002), as well as the local level of VEGF in hyperplastic epidermis is significantly increased in psoriasis, along with up-regulation of VEGFRs in ECs (Detmar *et al*., 1994; Bhushan *et al*., 1999). The observation that both

systemic and local cutaneous levels of VEGF are increased under psoriasis further warrants the need to investigate the relationship between systemic and local VEGF in skin disorders. The clinical characteristics of psoriasis, which are epidermal hyperplasia, inflammation, hyper-permeable blood vessels, and enlargement of lymphatic vessels (Christensen *et al*., 2006), are closely associated with bioactivity of VEGF. The contribution of VEGF to psoriatic pathogenesis has been confirmed in experimental models, where typical features of human psoriasis were observed in transgenic mice with increased VEGF levels (Detmar *et al*., 1998; Xia *et al*., 2003). Keratinocytic VEGF was induced by vasoactive intestinal peptide (VIP), which is specifically found in psoriatic epidermis (Kakurai *et al*., 2009; Yu *et al*., 2010). Interestingly, several studies reported that the susceptibility to psoriasis might be affected by VEGF genetic polymorphism. Single nucleotide polymorphism of the VEGF gene was found to be more frequent in patients with psoriasis compared to healthy individuals, with increased level of VEGF (Young *et al*., 2006). Promoter variations in the VEGF gene was also reported to be associated with development of psoriatic symptoms (Rogers and D'Amato, 2006), further supporting the role of VEGF in the etiology of psoriasis.

Phototoxicity

UV irradiation is a major physical stimulus to skin, causing cutaneous phototoxicity such as photo-irritation, photo-sensitization, photo-aging, and photo-carcinogenesis (Syed *et al*., 2012). UV exposure induces generation of reactive oxygen species (ROS) affecting cellular macromolecules and DNA, and also activates multiple signaling pathways responsible for cell growth and proliferation. Different signaling pathways are known to be activated by UVA (320-400 nm) and UVB (280-320 nm) (Mildner *et al*., 1999; Syed *et al*., 2012). VEGF expression in cultured keratinocytes was induced by UVB irradiation (Brauchle *et al*., 1996; Yano *et al*., 2005), both indirectly by releasing soluble factors such as IL-1 and TNF-α or directly by activating transcription factors such as NFκB, AP-1 or AP-2 (Blaudschun *et al*., 2000; Gille *et al*., 2000; Brenneisen *et al*., 2003). While UVB induced VEGF up-regulation in primary human keratinocytes and in immortalized keratinocytes (HaCaT) (Longuet-Perret *et al*., 1998; Brenneisen *et al*., 2003), UVA increased VEGF level only in HaCaT cells (Longuet-Perret *et al*., 1998; Gille *et al*., 2000) suggesting specific regulation of cutaneous VEGF signaling in the immortalized keratinocytes which may favor tumorigenic transformation. UV irradiation induces skin alteration such as erythema, hyper-permeability, edema, and epidermal hyperplasia, which are closely associated with VEGF. Hirakawa *et al*. (2005) demonstrated that VEGF promotes sensitivity to UVB-induced cutaneous photodamage in VEGF-transgenic mice, suggesting that VEGF may serve as a target for the prevention of photo-damage.

Skin cancinogenesis

It is well-established that tumor cells show increased metabolic demands and initiate angiogenic response. Consistently, VEGF up-regulation was frequently observed in skin cancers and it has been considered to act as an endothelial-specific mitogen (Detmar *et al*., 1995, Hicklin and Ellis, 2005). It is well known that VEGF regulates endothelial cells in tumor angiogenesis and vascular permeability, but it has been recently found that VEGF also plays an integral role in tumor cell signaling in autocrine manner, such as promoting dedifferentiation and an epithelial-mesenchymal transition (Senger, 2010; Cao *et al*., 2012; Goel and Mercurio, 2013). Other cells in tumor microenvironment including immune cells and fibroblasts can also be regulated by VEGF, enhancing tumorigenesis (Quail and Joyce, 2013). In skin cancer including squamous cell carcinomas, VEGF-VEGFR signaling was found to be critical for invasion, and selective VEGF over-expression was sufficient for tumor invasiveness *in vivo* (Detmar, 2000). Angiogenesis and lymphangiogenesis were required for tumor growth, metastasis, and further infiltration of inflammatory cells (Mantovani *et al*., 2008; Alitalo *et al*., 2013). Blockade of VEGF-C and D signaling resulted in suppressed inflammatory tumor microenvironment, leading to significant inhibition of early skin cancer progression (Alitalo *et al*., 2013). Interestingly, UVA specifically induced VEGF in HaCaT cells (Gille *et al*., 2000), suggesting that VEGF signaling may differ in premalignant phenotype.

VEGF INDUCTION BY XENOBIOTICS

Besides pathological skin conditions, keratinocyte-derived VEGF can be induced by several xenobiotics supporting its potential as a novel biomarker in chemical-induced skin problem. VEGF over-expression in keratinocytes was observed by the treatment with phorbol esters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA) (Diaz *et al*., 2000). It is also known that oxidants such as H_2O_2 can enhance VEGF expression in keratinocyte (Brauchle *et al*., 1996; Sen *et al*., 2002), suggesting that cutaneous VEGF can be regulated by redox control. Peroxisome proliferator-activated receptor-gamma (PPARγ) agonist troglitazone significantly induced VEGF expression in keratinocytes mediated by p38 MAPK activation (Schiefelbein *et al*., 2008). Of note, there is an initial study to suggest VEGF as a potential soluble mediator for hyper-permeability and inflammation, where several contact allergens, metals and an irritant induced VEGF in keratinocytes (Palacio *et al*., 1997). Still, the mechanisms and the roles of chemically induced VEGF in keratinocytes are largely unknown, which warrant future researches.

INVOLVEMENT OF VEGFR

Although VEGFRs are predominantly expressed in ECs supporting the paracrine signaling of keratinocyte-derived VEGF, all types of identified VEGFRs are also expressed in keratinocytes in normal epidermis (Man *et al*., 2006). The VEGFR signaling in keratinocytes involves in the proliferation and migration of normal keratinocytes (Man *et al*., 2006). Using VEGFR-1 specific neutralizing antibody, it was demonstrated that VEGFR-1 in keratinocytes promoted re-epithelialization through a novel autocrine pathway (Wilgus *et al*., 2005). Besides the constitutive expression in normal keratinocytes, VEGFRs are found to be functionally over-expressed in pathological condition including psoriatic epidermis (Man *et al*., 2006; 2008; Zhu *et al*., 2013), and can be up-regulated by UV irradiation (Zhu *et al*., 2012).

FUTURE DIRECTIONS

Identification of a biomarker for skin damage is an emerging issue for safety assessment of cosmetics or topically-administered medicinal compounds. Traditional skin toxicity test methods have been mostly performed in animal models. However, especially in cosmetic industries, the use of experimental animal is prohibited by the implementation of the 7th Amendment of the Cosmetic Directives in Europe (Directive 2003/15/EC), based on a growing attention on animal welfare. There have been intensive efforts to develop non-animal alternative tests for skin toxicity. The identification of reliable biomarkers for skin damage is prerequisite for development of new alternative methods. Also, a biomarker that can represent an integrated *in vivo* alteration would be useful to predict the potential biological effect and toxicity. Here we discussed the possibility of VEGF as a novel biomarker for keratinocytic damage in skin toxicity testing, based on the clear evidences on a significant role of VEGF in pathological alteration of keratinocytes under skin disorders.

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