The first peak of the UVB irradiation-dependent biphasic induction of vascular endothelial growth factor (VEGF) is due to phosphorylation of the epidermal growth factor receptor and independent of autocrine transforming growth factor α

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Abstract Ultraviolet B (UVB) irradiation, the major damaging component of sunlight, has earlier been reported to enhance cutaneous angiogenesis in chronically sun-exposed skin. We herein provide first evidence for a biphasic induction of the vascular endothelial growth factor (VEGF) following UVB irradiation of the human epidermal cell line HaCaT. The first VEGF peak occurred on mRNA level at 1 h and on protein level at 4 h postirradiation and is fully mediated by the UVB-dependent phosphorylation of the epidermal growth factor receptor, which subsequent to its phosphorylation also initiates at least in part the synthesis of transforming growth factor α that confers as shown previously the second late VEGF peak at 8 h on mRNA and at 24 h on protein level.

Key words: Ultraviolet B; Vascular endothelial growth factor; Epidermal growth factor receptor phosphorylation; PD 153035; Transforming growth factor α; HaCaT cell

1. Introduction

Chronically sun-exposed skin is characterized by widespread teleangiectasia, an increase in small blood vessels, and vascularized malignant tumors [1]. There is increasing evidence that the induction of angiogenesis precedes the formation of malignant tumors [2–6], suggesting that angiogenesis is not only crucial for tumor expansion, but also for the onset of malignancy. Among a variety of angiogenic growth factors, vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is a dimeric glycoprotein with a molecular weight of 34–45 kDa [7–11] that due to alternative splicing comprises different molecular isoforms [12,13]. VEGF induces microvascular hyperpermeability and represents a selective mitogen for endothelial cells [9,14]. VEGF exerts its biological effects by binding to its receptors VEGF-R1 (flt-1) and VEGF-R2 (flk/KDR) which are mainly expressed in endothelial cells [15–18]. Blocking of the VEGF function inhibits angiogenesis in the mouse cornea [19] and suppresses tumor growth and invasion [6,17,20–22]. Previous findings with ultraviolet B (UVB) induced up-regulation of VEGF in normal murine and human skin [23–25], in squamous cell carcinomas of sun-exposed anatomical sites [24,26,27] and suppression of UVB-induced angiogenesis in hairless mice upon intraperitoneal administration of neutralizing antibodies against VEGF supports the causal role of VEGF in angiogenesis after UVB irradiation in vivo. In further attempts to clarify the mechanistic base for the UVB induction of VEGF in more detail, most laboratories including ours [23,24,28,29] have found a late induction of VEGF protein synthesis and release occurring between 12 and 24 h postirradiation in a variety of epidermal cell lines and primary keratinocytes. In this study, we provide first evidence for a biphasic induction of VEGF on mRNA and protein level upon UVB irradiation of the human epidermal cell line HaCaT at a physiological dose of 10 mJ/cm^2. The first peak of $VEGF_{165}$ expression is due to the UVB-induced phosphorylation and activation of the epidermal growth factor receptor (EGF-R) and most likely initiates the earlier published release of transforming growth factor α (TGFα) starting at 6 h, which via autocrine binding and activation of the EGF-R is responsible for the second peak of VEGF release [28]. The identification of UVB-induced activation of the EGF-R underlying the first peak of VEGF release and its causal interrelation to the second VEGF peak in the biphasic UVB response may promote the development of selective, non-toxic inhibitors of the enhanced EGF-R function.

2. Materials and methods

2.1. Reagents

Cell culture medium Dulbecco's modified Eagle's medium (DMEM) and Trizol® reagent were from Life Technologies (Eggenstein, Germany) and fetal calf serum (FCS) from Biochrom (Berlin, Germany). All chemicals and biochemicals were obtained from Sigma (Deisenhofen, Germany) unless otherwise indicated. A mouse anti-human TGFα neutralizing antibody was purchased from Calbiochem (Bad Soden, Germany). Mouse anti-human EGF-R, mouse anti-phosphotyrosine and rabbit anti-mouse horseradish peroxidase (HRP) were from Santa Cruz Biotechnology (Heidelberg, Germany). The EGR-R-specific tyrosine kinase inhibitor PD 153035 was from Calbiochem (Bad Soden, Germany). $VEGF_{165}$ enzyme-linked immunosorbent assay (ELISA) kits were obtained by R&D Systems (Wiesbaden-Nor-

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Abbreviations: FGF, fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; EGF-R, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HRP, horseradish peroxidase; KGF, keratinocyte growth factor; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TGFα, transforming growth factor α; UV, ultraviolet; UVB, ultraviolet B; VEGF, vascular endothelial growth factor

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denstadt, Germany). Protein A agarose was from Santa Cruz Biotechnology (Heidelberg, Germany).

2.2. Cell culture

The human epidermal cell line HaCaT was cultured in DMEM supplemented with glutamine (2 mM), penicillin (400 U/ml), streptomycin (50 μg/ml), 10% FCS and grown on plastic tissue culture dishes in a humidified atmosphere of 5% CO_2 and 95% air at 37°C [30].

2.3. Light source and UVB irradiation

A 1000 W xenon high-pressure UV source was used in conjunction with a monochromator with holographic grating (Dermolum UMW, Fa. Müller, Moosinning, Germany). For the experiments, the total UVB spectrum (280–320 nm) was used. Fluences were determined by an integrated thermophile. The dose rates on the cell surface were 0.4 mW/cm^2 for the total UVB spectrum. To guarantee a constant intensity and spectral distribution, dosimetry and spectroradiometric analysis were performed prior to experiments with an OL-754 UV/visible light spectroradiometer (Optronic, Orlando, FL, USA). Subconfluent monolayer cultures of HaCaT cells were rinsed twice with phosphate-buffered saline (PBS) and thereafter irradiated under a thin layer of PBS at a dose of 10 or 50 mJ/cm^2 of the complete UVB spectrum [31]. There was negligible loss in viability of cell populations compared to cells held under similar conditions without irradiation. Following UVB irradiation, cells were replenished with their own medium or cultivated in DMEM without FCS for various periods of time. UVB doses applied in this study are relevant and easily reach basal keratinocytes in vivo [32].

2.4. ELISA

The cytokine ELISA assay was performed according to the manufacturer's protocol. Concentrations of the $VEGF_{165}$ protein were measured in cell culture supernatants using a quantitative sandwich ELISA (R&D Systems, Wiesbaden-Nordenstadt, Germany). Four hours before collecting the supernatants of the irradiated cells, the conditioned medium was replaced by serum-free DMEM. The concentrations of $VEGF_{165}$ to be tested in the samples were determined against standard curves using GraphPad™ software (San Diego, CA, USA). Means and S.D. were calculated, and the significance of the values was tested by the Student's *t* test or analysis of variance (ANOVA) (InStat software, GraphPad, San Diego, CA, USA).

2.5. Isolation of total RNA and RNase protection assay

Total RNA was extracted from HaCaT cells with Trizol® according to the manufacturer's recommendation (Life Technologies). The RNase protection assay was performed as published [33]. Densitometric analysis was performed using Scan Pack II software (Biometra, Göttingen, Germany).

2.6. Neutralization and inhibition studies

Two hours prior and directly after UVB irradiation at a dose of 10 mJ/cm^2, subconfluent HaCaT cells were incubated with neutralizing mouse anti-human antibodies specific for TGFα at concentrations of 100 and 500 ng/ml. For inhibition of EGF-R-mediated signal transduction, quiescent HaCaT cells were incubated with distinct concentrations of an inhibitor of tyrosine kinase phosphorylation highly specific for the EGF-R, 4-[(bromophenyl)amino]-6,7-dimethoxyquinazoline (PD 153035, Calbiochem, Bad Soden, Germany), 2 h prior to and 4 h after UVB irradiation at a dose of 10 mJ/cm^2. Four hours after UVB irradiation, the supernatants were collected for determination of $VEGF_{165}$ protein levels.

2.7. Analysis of EGF-R tyrosine phosphorylation

Subconfluent HaCaT cell cultures were washed twice with ice-cold PBS and scraped into 0.5 ml of lysis buffer (20 mM imidazole, 100 mM KCl, 1 mM $MgCl_2$, 200 mM EGTA, 0.5% Triton X-100, 150 nM aprotinin, 2 μM leupeptin, 1.5 μM pepstatin A, 1 mM PMSF, 10 mM NaF, 200 mM $Na_2MoO_4{\cdot}2H_2O$, 1 mM Na_3VO_4 (pH 10)). After sonification, 800 μl of cellular lysate (protein concentration of 1.25 μg/μl) was precleared with protein A agarose (Santa Cruz Biotechnology, Heidelberg, Germany) for 1 h at 4°C. Immunoprecipitation was performed overnight at 4°C using a monoclonal antibody specific for human EGF-R (Santa Cruz Biotechnology, Heidelberg, Germany). The protein A agarose pellets were washed three times with Tris-buffered saline (TBS) (4°C), boiled in 60 μl of 2×sample buffer (100 mM Tris–HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 0.2% bromophenol blue, 20% glycerol, 5% 2-mercaptoethanol) for 5 min and the proteins were resolved by SDS–PAGE. Proteins were transferred to Protran nitrocellulose membrane (Schleicher and Schüll, Dassel, Germany), and the membrane was blocked in 1% blocking buffer (1% casein, 1% polyvinylpyrrolidone in TBS (pH 7.2)) for 1 h. For immunodetection of phosphorylated EGF-R, an anti-phosphotyrosine antibody (PY99, Santa Cruz Biotechnology, Heidelberg, Germany) was added to 0.5% blocking buffer plus 0.1% PEG-6000 at a concentration of 0.1 μg/ml. Staining was performed with a HRP-linked antibody (Santa Cruz Biotechnology, Heidelberg, Germany) which was visualized using Luminol ECL detection reagent (Santa Cruz Biotechnology, Heidelberg, Germany). Densitometric analysis was performed using Scan Pack II software (Biometra, Göttingen, Germany).

2.8. Cytotoxicity assay

The viability of HaCaT cells was measured 24 h after UVB irradiation at different doses. 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma, Deisenhofen, Germany) was used for the quantification of living metabolically active cells. Mitochondrial dehydrogenases metabolize MTT to purple formazan dye [34]. Cytotoxicity was calculated as percent of formazan formation in cells which have been irradiated compared to mock-treated cells.

3. Results and discussion

3.1. Biphasic induction of the VEGF upon UVB irradiation of HaCaT cells

Recently, several laboratories reported on the induction of VEGF in human epidermal cell lines (A431, HaCaT) and human primary keratinocytes in vitro and in vivo following UVB irradiation [23,24,28,29]. These studies including our own [28] reported on the monophasic induction of VEGF mRNA and protein levels occurring between 4 and 24 h post-irradiation. An exact time-dependent response curve has, however, not yet been established. Using a highly specific sandwich ELISA, we have monitored the protein level of the $VEGF_{165}$ isoform at different time points postirradiation. Exposure of HaCaT cells to a low UVB dose of 10 mJ/cm^2 resulted in a first peak with a 2.3-fold increase in the specific $VEGF_{165}$ protein concentration at 4 h postirradiation, while a second 4-fold increase was observed at 24 h postirradiation (Fig. 1a). At the used UVB dose of 10 mJ/cm^2, $>80\%$ of the HaCaT cells were viable 24 h postirradiation (Fig. 1a, inset). Interestingly, this biphasic response $VEGF_{165}$ protein concentration was also reflected on mRNA level as determined by a previously established RNase protection assay [23] with subsequent densitometric analysis. Exposure of HaCaT cells to a UVB dose of 10 mJ/cm^2 resulted in a first peak of specific $VEGF_{165}$ mRNA level with a 1.8-fold induction already 1 h postirradiation and a second increase in $VEGF_{165}$ mRNA level of 2.3-fold was observed at 12 h when compared to mock-treated control cells. Similar results were obtained for specific mRNA levels of $VEGF_{121}$ (Fig. 1b). These data indicate a biphasic response of VEGF synthesis on mRNA and protein level following UVB irradiation. Our data, as based on the specific ELISA for $VEGF_{165}$, do not allow to monitor the contribution of cell surface-associated VEGF isoforms to the overall VEGF levels upon exposure of HaCaT cells to UVB irradiation. There is, however, evidence that UVB irradiation most likely via the proteolytic direct cleavage of the cell-associated $VEGF_{189}$ isoform by the urokinase-type plasminogen activator (uPA) and via proteolytic conversion of cell surface bound plasminogen to plasmin and subsequent cleavage of cell surface-associated $VEGF_{189}$ and $VEGF_{165}$ [35,36]

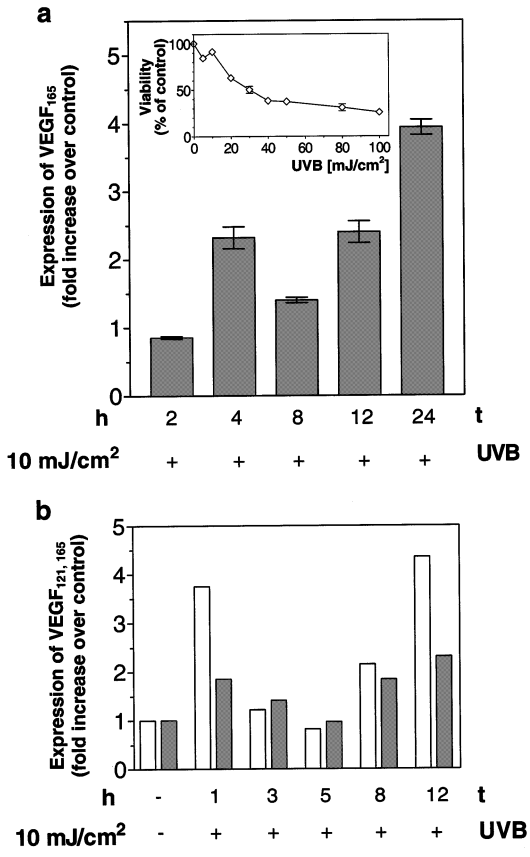

Fig. 1. VEGF is biphasically up-regulated on protein and mRNA level after UVB irradiation. (a) Subconfluent quiescent HaCaT cells were UVB-irradiated at a dose of 10 mJ/cm^2 and the $VEGF_{165}$ protein contents of the supernatants were analyzed using a quantitative sandwich ELISA specific for $VEGF_{165}$. Conditioned medium from mock-irradiated cells served as a control. Data are expressed as fold increase over mock-treated controls which were set one (mean ± S.D., $n = 3$). The inset in (a) depicts the UVB dose-dependent decrease in cell viability at 24 h postirradiation as measured by the MTT cytotoxicity assay further detailed in Section 2. (b) Subconfluent quiescent HaCaT cells were UVB-irradiated at a dose of 10 mJ/cm^2 and VEGF mRNA expression for the isoforms $VEGF_{121}$ (□) and $VEGF_{165}$ (■) was analyzed by means of RNase protection assay at the indicated time points. Total RNA from mock-irradiated cells served as a control. Densitometric analysis was performed using Scan Pack II software (Biometra, Göttingen, Germany).

may contribute to the release of the cell surface bound VEGF isoforms. This interesting question awaits further elucidation in an independent study.

3.2. UVB irradiation results in a time-dependent increase in phosphorylation of the EGF-R

UVB irradiation has been reported to be able to phosphorylate and activate the EGF-R in the absence of ligands in the cervix carcinoma cell line HeLa [37]. This UVB-induced tyrosine phosphorylation reflects EGF-R activation and leads to the recruitment of adaptor proteins and other postreceptor events [38]. Also, EGF-R phosphorylation was found in human keratinocytes after exposure to UVB [39]. In order to analyze a potential regulatory role of EGF-R phosphorylation, which may be responsible for the first peak of the UVB-dependent biphasic VEGF induction, we first established a time kinetic of EGF-R phosphorylation upon UVB irradiation (Fig. 2). Already 5 min postirradiation, a 3.3-fold

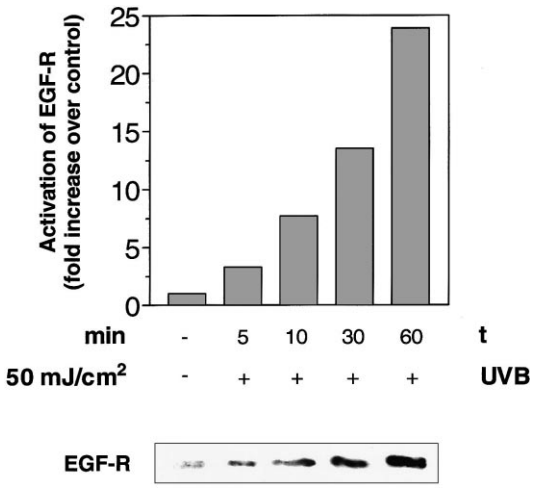

Fig. 2. The EGF-R is activated by UVB irradiation. Subconfluent quiescent HaCaT cells were irradiated at a dose of 50 mJ/cm^2 of the complete UVB spectrum. Cellular lysates were subjected to EGF-R phosphorylation analysis at different time points thereafter. Densitometric analysis was performed using Scan Pack II software (Biometra, Göttingen, Germany). The blot is representative of two independently performed experiments.

with a further time-dependent increase in EGF-R phosphorylation of up to 24-fold at 60 min postirradiation was observed (Fig. 2). All following experiments which aimed at the inhibition of EGF-R phosphorylation were performed in the presence of the specific inhibitor PD 153035 [40,41] 2 h prior to UVB irradiation and during a 4 h postirradiation period. Similar to our data, a sustained phosphorylated state

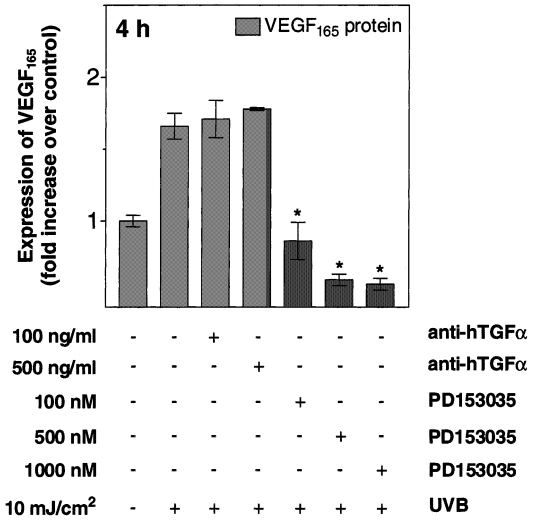

Fig. 3. The first UVB-induced peak of $VEGF_{165}$ protein is due to EGF-R phosphorylation and independent of TGFα. Incubation of HaCaT cells 2 h prior to and 4 h after UVB irradiation (10 mJ/cm^2) with neutralizing antibodies specific for hTGFα and subsequent analysis of $VEGF_{165}$ protein in supernatants collected at 4 h postirradiation did not result in any reduction in $VEGF_{165}$ protein concentrations. Incubation of HaCaT cells with distinct concentrations of 4-[(bromophenyl)amino]-6,7-dimethoxyquinazoline (PD 153035), a specific inhibitor of the tyrosine kinase activity of the EGF-R, 2 h prior to and 4 h after UVB irradiation significantly reduced $VEGF_{165}$ protein levels ($^*P < 0.0001$ compared to UVB-irradiated cells without PD 153035; ANOVA). Data are expressed as fold increase over mock-treated control which was set one (mean ± S.D.). Three independent experiments were performed.

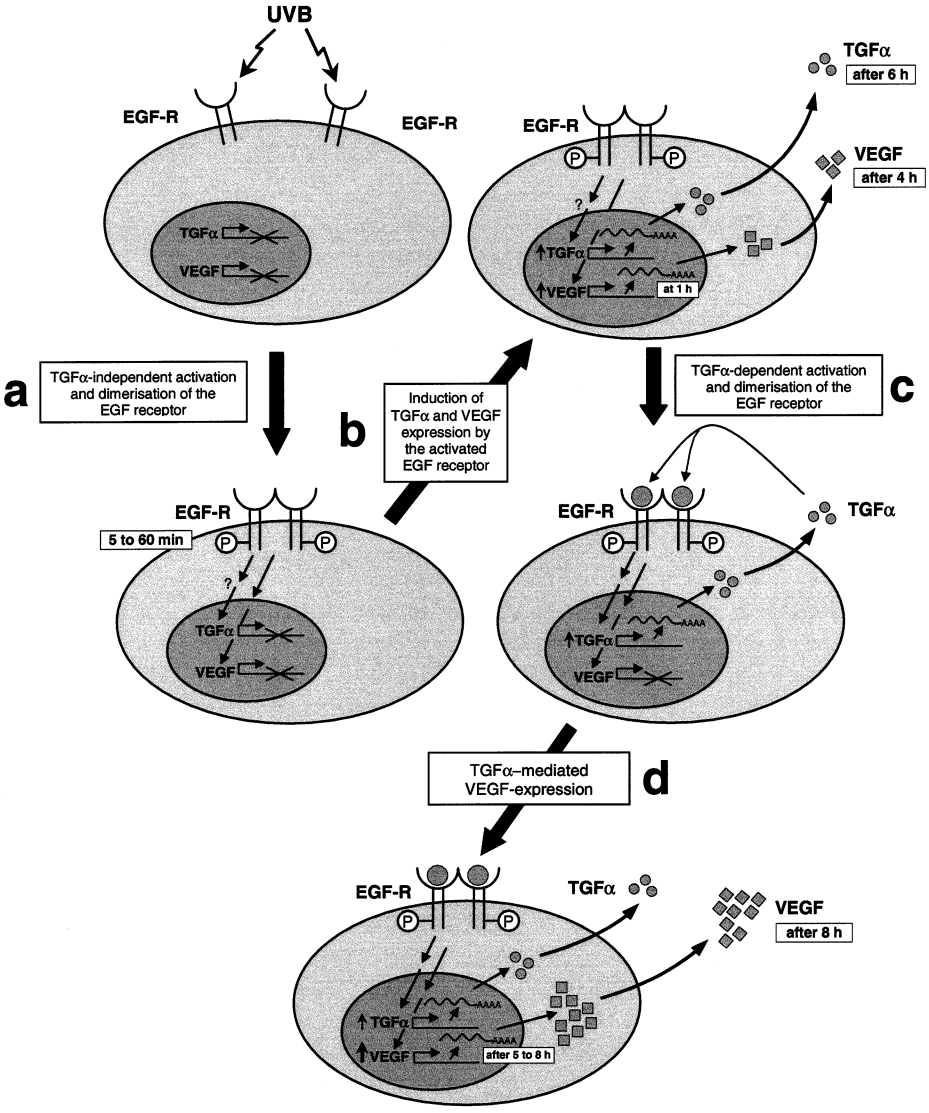

Fig. 4. Molecular mechanisms underlying the biphasic $VEGF_{165}$ induction after UVB irradiation. UVB irradiation of HaCaT cells leads to a TGFα-independent phosphorylation and activation of the EGF receptor (a). This activation results in an early peak of $VEGF_{165}$ on mRNA and protein level and in addition is responsible for the release of TGFα 6 h postirradiation. Whether the TGFα release is due to TGFα mRNA synthesis or to preformed TGFα has not been investigated (b). Secreted TGFα subsequently binds to the EGF receptor and leads to a TGFα-dependent activation of the EGF receptor (c). After this TGFα-dependent EGF-R activation, a second induction in the synthesis of $VEGF_{165}$ occurs at 8–24 h after UVB irradiation (d). All time points included in the diagram refer to time point 0 directly after UVB irradiation.

of the EGF-R upon UVB irradiation has earlier been reported [39,42]. The study by Peus et al. revealed that the phosphorylated state of the EGF-R was still enhanced 2 h postirradiation of human primary keratinocytes at a dose of 40 mJ/cm^2. So far, the underlying molecular mechanisms of the sustained EGF-R phosphorylation following UVB irradiation are not entirely clear. There is, however, evidence that UV irradiation reduces the activity of tyrosyl phosphatases which ultimately control the phosphorylation state of receptors and proteins [43,44].

3.3. *The first peak of the biphasic induction of $VEGF_{165}$ after UVB irradiation is due to specific phosphorylation and activation of the EGF-R and, in contrast to the second VEGF peak, independent of autocrine TGFα*

We previously reported that the second late peak of $VEGF_{165}$ induction depends at least in part on the UVB-induced phosphorylation of the EGF-R with subsequent release of TGFα, which, as judged from the complete abrogation of the second peak by TGFα neutralizing antibodies, is fully responsible for this second VEGF peak upon UVB irradiation [28].

In previous experiments (Blaudschun et al., to be published elsewhere), a time-course analysis demonstrates that at 6 h postirradiation, but not earlier, a significant induction of TGFα occurred after exposure of HaCaT cells at a UVB dose of 10 mJ/cm^2. To further establish whether the UVB-dependent herein described first peak of VEGF synthesis, similar to the second VEGF peak, is mediated by TGFα, neutralizing antibody experiments were conducted. This was to exclude the possibility that even minor induction of TGFα at time points before 6 h postirradiation, which may not be detected by the TGFα ELISA, contribute to the first UVB-induced VEGF peak. By contrast to the complete abrogation

of the second late peak of VEGF induction with neutralizing antibodies against TGFα at a concentration of 100 ng/ml [28], we were unable to reduce the first VEGF peak following UVB irradiation with TGFα neutralizing antibodies even at a concentration clearly exceeding 100 ng/ml (Fig. 3), indicating that the first peak of the biphasic VEGF induction following UVB irradiation is completely independent of TGFα. As the phosphorylation of the EGF-R occurred as early as 5 min post UVB irradiation, we studied the potential role of this initial EGF-R phosphorylation in mediating the UVB-dependent first VEGF peak.

For this purpose, quiescent HaCaT cells were incubated with an inhibitor of tyrosine kinase phosphorylation highly specific for the EGF-R, 4-[(bromophenyl)amino]-6,7-dimethoxyquinazoline (PD 153035) [40,41] 2 h prior to and 4 h after UVB irradiation. Interestingly, the first $VEGF_{165}$ peak following UVB irradiation could be completely abrogated to constitutive $VEGF_{165}$ levels at a non-toxic PD 153035 concentration of 100 nM. The same PD 153035 concentration did not completely inhibit but reduced the UVB-dependent TGFα induction to 30% and that of $VEGF_{165}$ to 35% of the corresponding VEGF induction in UVB-treated cells at 24 h postirradiation (Blaudschun et al., submitted). These data suggest that the initial UVB-dependent EGF-R phosphorylation is responsible (I) for the first VEGF peak and (II) is partly responsible for the TGFα release which, via binding and activation of the EGF-R, contributes to the second VEGF peak (Fig. 4). In fact, binding of TGFα to the EGF-R has earlier been reported to stimulate its phosphorylation with subsequent release of TGFα [45,46]. Currently, the mechanisms which, in addition to the initial EGF-R phosphorylation, contribute to the increased TGFα release and the second VEGF peak after UVB irradiation are unclear. Other UVB-induced cytokines or receptors with autocrine TGFα-inducing properties may play a role [47,48]. In this regard, keratinocyte growth factor (KGF) receptor ligands like KGF and acidic fibroblast growth factor (FGF) have been reported to induce TGFα expression and activate the EGF-R signalling pathway in cultured epidermal keratinocytes [47]. Furthermore, tumor necrosis factor α regulates TGFα expression in hepatocytes [48]. Alternatively, it might be possible that TGFα acts via a receptor, which, distinct from the EGF-R, leads to the induction of the second VEGF peak.

Regardless of the exact mechanism, we here demonstrate a biphasic response of VEGF induction upon UVB irradiation at a physiological dose. Accordingly, the dose that stimulates VEGF expression in human keratinocytes or HaCaT cells (10 mJ/cm^2) is equivalent to the dose of UVB irradiation reached at the skin surface after 1 min of sun exposure on a clear day in July at noon at the latitude between 48 and 56 degrees North [49,50]. In the here reported experiments, HaCaT cells were directly irradiated. However, normal skin keratinocytes are protected by the stratum corneum. Correction of the wavelength efficiencies (295–320 nm) determined in vitro with the transmission spectrum of the overlying stratum corneum and the upper epidermal cell layers [51] suggests that about 6–18% of the incident UVB dose at the skin surface reaches the keratinocytes beneath the stratum corneum. Therefore, VEGF induction doses in vivo would require approximately 6–18 min of sun exposure. This time range applies for human sunbathing and is easily acquired during a sunny summer day.

Even though we were recently able to show a causal role of VEGF in UVB-induced angiogenesis in vivo (Blaudschun et al., to be published elsewhere), it is most likely that apart from VEGF, other factors are involved in the angiogenic UVB response in vivo. In fact, basic FGF, a positive angiogenic molecule, has been found to be induced upon UVB irradiation of murine skin [52].

The biphasic UVB-induced VEGF expression is apparently mediated by different though interrelated mechanisms. Accordingly, we found that the first peak of the biphasic VEGF induction upon UVB irradiation is regulated by the specific phosphorylation and activation of the EGF-R and, in contrast to the second VEGF peak, is independent of TGFα. These data together with our previous findings (Blaudschun et al., to be published elsewhere) indicate that the initial phosphorylation of the EGF-R occurring as early as 5 min postirradiation apparently, via the TGFα release at 6 h postirradiation with subsequent binding and activation of the EGF-R, accounts at least in part for the observed second VEGF peak.

Collectively, we here provide a detailed molecular description for the regulation of VEGF that different from earlier published results [53–55] may reflect critical steps in UV-induced angiogenesis in physiological and pathological conditions.

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