Essential role of an activator protein-2 (AP-2)/specificity protein 1 (Sp1) cluster in the UVB-mediated induction of the human vascular endothelial growth factor in HaCaT keratinocytes

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Chronic sun exposure of the skin has long been postulated to enhance cutaneous angiogenesis, resulting in highly vascularized skin cancers. As the UVB component of sunlight is a major contributor to photocarcinogenesis, we aimed to explore the effects of UVB radiation on vascular endothelial growth factor (VEGF) gene expression, using the immortalized keratinocyte cell line HaCaT as a model for transformed premalignant epithelial cells. In the present paper, we studied the molecular mechanism of UVB-induced VEGF providing a major angiogenic activity in tumour progression and invasion. After 12–24 h of UVB irradiation, a 2.4- to 2.7-fold increase in endogenous VEGF protein level was measured, correlating with an up to 2.5-fold induction of promoter-based reporter gene constructs of VEGF. Furthermore, we identified a GC-rich UVB-responsive region between –87 and –65 bp of the VEGF promoter. In electrophoretic mobility-shift assays, this region binds Sp1-

dependent protein complexes constitutively and an additional UVB-inducible protein complex distinct from Sp1 protein. The transcription factor AP-2 (activator protein-2) was detected as a component of the UVB-inducible protein complex. The critical role of the AP-2/Sp1 (specificity protein 1) cluster was supported by demonstration of a significant reduction of UVB-mediated promoter activity upon deletion of this recognition site. The specificity of this region for UVB irradiation was demonstrated using PMA, which increased VEGF activity in HaCaT cells after transient transfection of the deleted promoter construct.

In conclusion, our data clarified regulatory mechanisms of UVB-dependent VEGF stimulation which may be critical for angiogenic processes in the skin.

Key words: angiogenesis, *in vitro* mutagenesis, photocarcinogenesis, promoter construct.

INTRODUCTION

UV irradiation is an important environmental stimulus and carcinogen, tilting the balance of normal skin homeostasis to several pathologies [1]. Besides causing permanent genetic changes, involving proto-oncogenes and tumour suppressor genes, such as p53 [2], UV irradiation may induce auto- and paracrine cytokines with subsequent activation of signal transduction pathways that are related to growth, tissue degradation [1] and possibly angiogenesis. Chronically sun-exposed skin is characterized by widespread telangiectasia, an increase in small blood vessels, and vascularized malignant skin tumours [3]. There is increasing evidence that the induction of angiogenesis precedes the formation of malignant tumours [4,5], suggesting that angiogenesis is crucial not only for tumour expansion, but also for the onset of malignancy. In this respect, the number of blood vessels infiltrating primary tumours provides a prognostic indication of the disease status [6]. Among a variety of angiogenic growth factors, vascular endothelial growth factor (VEGF) is a highly conserved disulphide-bonded dimeric glycoprotein with a molecular mass of 34 to 45 kDa [7,8]. The VEGF family comprises several polypeptides that arise from alternative splicing of a single gene [9]. VEGF expression appears to be highly susceptible to hypoxia [10], or exogenous and endogenous

cytokines and growth factors respectively, for example interleukin (IL)-1 and epidermal growth factor (EGF) [11], IL-6 [12], tumour necrosis factor α (TNF α) [13], transforming growth factor (TGF) β [14] and TGF α [15]. 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 [16], thus acting as a selective endothelial cell mitogen. Overexpression of VEGF in different cells has been reported in certain non-neoplastic processes characterized by increased microvascular permeability and angiogenesis, e.g. the development and differentiation of the cardiovascular system [17], wound healing [18], and psoriasis [19]. In addition, VEGF has been reported to be a major mediator of tumour angiogenesis [20], and the *in vivo* relevance of VEGF as a paracrine inducer of cutaneous vessels was recently demonstrated in a study in which skh-1 hairless mice were exposed to repetitive UVB irradiation [21]. Blocking VEGF function suppresses tumour growth and invasion [5,22].

In view of the growing evidence that the increase in UVB irradiation (280–320 nm) on the earth's surface due to stratospheric ozone depletion [23,24] represents a major environmental threat to the skin [25], we have focused our studies on the regulation of the UVB-initiated induction of VEGF in the immortalized human epidermal cell line HaCaT [26]. HaCaT cells may be classified as premalignant cells because immortaliza-

Abbreviations used: EGF, epidermal growth factor; EMSA, electrophoretic mobility-shift assay; IL, interleukin; MTR, mithramycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TGF, transforming growth factor; TNF α , tumour necrosis factor α ; VEGF, vascular endothelial growth factor.

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tion is considered to be an essential step in experimental carcinogenesis [27]. Whereas the UVA (320–400 nm)-mediated induction of HaCaT cells [28] has been reported to be modulated in a fashion comparable with that seen with TGF α [29], understanding of the molecular mechanisms underlying the UVB-mediated up-regulation of VEGF is still limited. In previous years it has been demonstrated that UVB irradiation increases the VEGF level in HaCaT keratinocytes [28,30]. More recently, we reported a biphasic induction of VEGF following UVB irradiation of the HaCaT cells. This induction is regulated in a co-ordinated fashion by activation of the EGF receptor and subsequent time-delayed release of TGF α , which is responsible for the second VEGF peak [31]. These findings [15,29,31] led us to hypothesize that similar recognition sites in the VEGF promoter region might be, at least in part, responsible for the UVB-mediated second peak of VEGF at 8–24 h after irradiation.

In the present study, we show that a GC-rich TGF α -responsive region at –87 to –65 bp, with respect to the transcription start site of the human VEGF promoter containing two specificity protein 1 (Sp1) recognition sites and an overlapping activator protein-2 (AP-2) site, are necessary for the UVB-mediated VEGF response. AP-2 transcription factor was detected as an essential component of the UVB-inducible DNA binding complex. Conclusively, our observations provide evidence that AP-2-dependent DNA binding is required for UVB-mediated expression of VEGF.

MATERIALS AND METHODS

HaCaT cell cultures

The human immortalized keratinocyte cell line HaCaT [utilized between passages 66 and 78; generously provided by Dr. Norbert E. Fusenig, German Cancer Research Center (DKFZ), Heidelberg] was grown in Dulbecco's modified Eagle's medium (Life Technologies, Eggenstein, Germany) supplemented with 10% fetal calf serum (Biocrom, Berlin, Germany), penicillin (400 units/ml), streptomycin (50 μ g/ml) and glutamine (2 mM) at a temperature of 37 °C and an atmosphere of 5% CO₂ and 95% O₂, as described elsewhere [32]. The cells were routinely passaged twice a week.

Prior to UVB irradiation or pretreatment with other chemical compounds, confluent cell monolayers were cultured in serum-free medium for about 18 h.

Light source and UV irradiation

For UVB irradiation a xenon high-pressure UV source was used in conjunction with a monochromator with holographic grating (Dermolum UMW, Müller, Moosinning, Germany) emitting a continuous spectrum between 280 and 320 nm [33]. 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 (Optronics, Orlando, FL, U.S.A.). Fluence rate at the cell surface following irradiation was 0.4 mW/cm². Initially, the UVB doses employed ranged from 5 to 100 mJ/cm² for the cytotoxicity assays, and for subsequent experiments, 10 mJ UVB/cm² were applied. There was negligible loss in the viability of cell populations compared with cells held under similar conditions without irradiation. The UVB doses applied in this study are relevant and can easily reach basal keratinocytes *in vivo* [34]. For gel mobility shift analysis, cells were exposed to UVB (50 mJ/cm²) or UVA radiation (10 J/cm²) utilizing the UV801KL device (Waldmann Medizintechnik, Villingen-Schwenningen, Germany). UVA wavelengths ranged from 320 nm to 400 nm, exhibiting a narrow-range

maximum at 365 nm. The fluence rate at the sample surface was approx. 1.4 mW/cm² within the UVB spectrum, and 8.6 mW/cm² within the UVA spectrum. For UVA and UVB irradiation, confluent and serum-starved HaCaT cells were irradiated while under a thin layer of PBS, and replenished with their own (conditioned) medium thereafter. To study time kinetics of VEGF expression, the medium was replaced by fresh medium without serum and after an additional 4 h, the supernatants were collected and used in ELISA assays.

Cytotoxicity assay and viability

The viability of non-transfected and transfected HaCaT cells was measured 24 h after UVB irradiation alone or in combination with mithramycin (MTR), an antitumour antibiotic known to specifically interact with GC-rich regions of DNA non-intercalatively [35], and which was added to the cells 2 h prior to and for a further 2 h after irradiation. For the quantification of living metabolically active cells 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, Deisenhofen, Germany) was used. Mitochondrial dehydrogenases metabolize MTT to form a purple formazan dye [36]. Cytotoxicity was calculated as percent of formazan formation in irradiated cells compared with mock-treated cells.

Measurements of VEGF₁₆₅ protein levels: ELISA

VEGF₁₆₅, the secreted predominant VEGF isoform in humans derived from alternative splicing [37], was measured in cell culture supernatants by means of sandwich ELISAs (R&D Systems, Wiesbaden, Germany) according to the manufacturer's recommendations, using pre-coated 96-well immunoplates, goat anti-human polyclonal VEGF antibody, and anti-goat horseradish peroxidase conjugate. 3,3',5,5'-Tetramethylbenzidine was used as a peroxidase substrate and optical densities were read at 450 nm. Concentrations of VEGF₁₆₅ protein were determined against standard curves using GraphPadTM software (San Diego, CA, U.S.A.).

Plasmid constructs and transient transfection

A 3.2 kb Kpn I–Nar I fragment of the genomic DNA clone from the human VEGF gene [9] which had been subcloned into the promoterless luciferase reporter vector pGL2-basic (Promega, Heidelberg, Germany) and a series of deletion derivatives [38], were used as shown in Figures 4 and 5. The UVB-responsiveness of these constructs was tested by using transient transfection assays. Co-transfections with the β -galactosidase gene containing pCMV- β Gal Vector (Clontech, Heidelberg, Germany) were performed to standardize transfection efficiency. Plasmids were transfected into HaCaT cells using the LipofectAMINETM reagent (Life Technologies) following the manufacturer's protocol with minor modifications. Briefly, 24 h after transfection, cells were washed twice with PBS and cultured in serum-free medium for 18 h. After extensive washing with PBS, cells were UVB-irradiated and control cells were mock-treated and incubated for 12 h in fresh serum-free medium supplemented with 0.2% lactalbumin hydrolysate (Sigma). Cells were washed twice in ice-cold PBS, detached and then lysed in 150 μ l of reporter lysis buffer (Promega). Aliquots (70 μ l) of total cellular protein were measured for luciferase activity with a luciferase assay system (Promega), according to the manufacturer's instructions. For quantification of the β -galactosidase activity 5 μ l of a 1:10 dilution of total cellular protein was used in a chemiluminescent reporter assay following the manufacturer's protocol. Measure-

ments of luciferase and β -galactosidase activity were performed in a type 2010 luminometer (ALL, San Diego, CA, U.S.A.).

In vitro mutagenesis of VEGF promoter constructs

In vitro mutagenesis was used to introduce a deletion of 27 bp into the full-length VEGF promoter construct (3.2 kb *KpnI*–*NarI* fragment) and into the shortest promoter construct (181 bp *ApaI*–*NheI* fragment). The deletion comprises three (–94 to –68 region with respect to the transcription start site, identical to –1133 to –1107 promoter region with respect to translation start site [9]) out of four Sp1 sites, including an overlapping AP-2 site in direct association with the transcription start site of the VEGF promoter. Mutagenesis was performed in a two-step overlap PCR amplification procedure, as previously described [39]. For each construct, four primers were designed and synthesized (Eurogentec, Cologne, Germany) to provide two different PCR products with a region of common sequence. The following primers were used for mutation of the full-length promoter construct (3.2 kb *KpnI*–*NarI* fragment): 5'-ATTCTCGAGACCCCCATTCTATTTCAGAAGATGA-3' (a1), 5'-CGGGACCGGACAGGCGAGCCTCAG-3' (b), 5'-CCTGTC-CGGTCCCGGGCGGGC-3' (c), 5'-ATAAGCTTAAGAAGA-GGGATAAAACCCGGATC-3' (d). For modification of the shortest promoter construct (181 bp *ApaI*–*NheI* fragment) the primers 5'-AATTCTCGAGATGGGTAATTTTCAGGCTGT-GAAC-3' (a2), b, c and d were used. In subsequent reactions, two overlapping subfragments of the target sequence were amplified to obtain the mutated constructs VEGF.1Δ3SP-1/AP-2 and VEGF.9Δ3SP-1/AP-2. For each reaction a flanking primer (a1, a2 or d) and an internal primer (b or c) containing sequences from each side of the region to be deleted were used. A 1.7 kb *Bam*HI fragment of the VEGF promoter region served as a PCR template. The mutated PCR products were cloned into the PCR cloning vector pT-Adv (Clontech) and the introduced deletions were confirmed by sequence analysis.

Preparation of nuclear extracts and electrophoretic mobility-shift assay (EMSA) analysis

Confluent HaCaT cultures were mock-irradiated or exposed to UVB (50 mJ/cm²) or to UVA (10 J/cm²). Nuclear proteins were extracted after 1 h (including the time of irradiation), as previously described [15]. The oligonucleotide was synthesized to span the region between –87 bp and –65 bp of the human VEGF promoter with respect to the transcription start site and identical to –1126 to –1105 of the promoter region with respect to translation start site, containing two Sp1 and an overlapping AP-2 site: 5'-TTTCCGGGGCGGGCCGGGGGCGG-GGTAT-3'. The underlined sequence served as a template for the synthesis of the second strand. Radiolabelled double-stranded DNA was synthesized by annealing an oligonucleotide complementary to the underlined sequence listed above (5'-ATACC-CCGC-3'), and by extension of the second strand with Klenow fragment, 50 μ Ci of [α -³²P]dCTP, unlabelled dATP, dGTP and dTTP. Unincorporated nucleotides were removed by column chromatography. The DNA binding reaction was performed for 30 min at room temperature in a volume of 20 μ l, containing 5 μ g of nuclear protein extract, 2.5 mg/ml BSA, 105 c.p.m. α -³²P-labelled probe (approx. 0.5–1.0 ng), 0.1 mg/ml poly [dI:dC] (Sigma), 5 μ l of 4x binding buffer [1x buffer: 10 mM TrisCl (pH 7.8), 100 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 10% (v/v) glycerol, 1 mM DTT] with or without 100-fold molar excess of unlabelled competitor. Competitor (Sp1 consensus oligonucleotides, Promega) and specific AP-2 and p65/Rel (Santa Cruz Biotechnology, Heidelberg, Germany) antibodies were

added 15 min prior to adding the probe. Samples were subjected to electrophoresis on a native 4% polyacrylamide gel for 3–4 h at 100 V.

RESULTS

Cytotoxicity of UVB irradiation and the antitumour antibiotic MTR

In order to avoid interference of cytotoxicity from UVB irradiation and/or MTR treatment, we performed the MTT assay to determine the optimal non-toxic concentrations at which more than 75% of the HaCaT keratinocytes survived at least 24 h after irradiation or incubation with no change in morphology (Figure 1). Irradiation of non-transfected and transfected HaCaT cells with the total UVB spectrum (280–320 nm) at doses $\leq$ 15 mJ/cm² satisfied this requirement. More than 80% of HaCaT cells were viable after incubation with increasing concentrations of MTR up to 100 nM and at a constant dose of 10 mJ UVB/cm² (Figure 1). Thus for subsequent experiments, HaCaT keratinocytes were UVB irradiated at a dose of 10 mJ/cm² and/or treated at a concentration of 50 nM MTR.

UVB irradiation results in a second VEGF protein peak in HaCaT cells

It was conclusively demonstrated that, in HaCaT cells, UVB irradiation led to a delayed release of TGF α . Although TGF α was not involved in the first VEGF₁₆₅ peak [31], it seemed to be, at least in part, responsible for a second VEGF₁₆₅ protein secretion at a later time point after irradiation. Therefore confluent and serum-starved HaCaT keratinocytes at low passage numbers were tested for their ability to show this second VEGF₁₆₅ peak (Figure 2). The cells were irradiated at a UVB dose of 10 mJ/cm² and supernatants were checked for the occurrence of VEGF₁₆₅ protein at different time points post-irradiation. Exposure of HaCaT cells to the low UVB dose resulted in a second increase in VEGF₁₆₅ protein level starting at 8 h post-irradiation, but the peak of this increase was reached at 12 to 24 h upon irradiation with a 2.4-fold increase at 12 h and a 2.7-fold increase at 24 h (Figure 2). This increase in VEGF protein level correlates with the 2.3-fold induction of the endogenous VEGF gene at 12 h post-irradiation, as reported earlier [31] using the RNase

Figure 1 Cytotoxicity of UVB irradiation and the antitumour antibiotic MTR

Confluent non-transfected (●) or transfected (○) HaCaT cells were irradiated with different doses of the total UVB spectrum (280–320 nm) or incubated with increasing concentrations of MTR in combination with a constant dose of 10 mJ UVB/cm² (□). The percentage of living cells in medium without fetal calf serum was measured 24 h after treatment. Values represent mean $\pm$ standard deviation ($n = 3$).

Figure 2 Increase in UVB-induced VEGF protein expression occurs in a time-dependent manner

Confluent, serum-starved HaCaT cells were UVB irradiated at a dose of 10 mJ/cm² and at after 8 h (A), 12 h (B), and 24 h (C) VEGF₁₆₅ protein levels were measured by sandwich ELISA. Data are expressed as pg of VEGF × 10⁻²/μg total protein (mean ± S.D.; grey bars) and as fold increase over unirradiated controls (0) which were set at 1.0 (white bars). **P* = 0.0052 (A), #*P* = 0.0002 (B), and §*P* = 0.0044 (C) compared with mock-irradiated controls (Student's *t* test). Three independent experiments were performed. S.D., standard deviation.

protection assay. At 48 h after UVB irradiation, the VEGF expression in HaCaT keratinocytes was down-regulated again (results not shown). These results demonstrated that the UVB-mediated stimulation of VEGF expression did not depend on a

Figure 3 MTR inhibits the VEGF gene expression upon UVB irradiation of HaCaT keratinocytes

Confluent HaCaT cells were incubated prior to and after UVB irradiation at a dose of 10 mJ/cm². VEGF₁₆₅ protein levels were measured 24 h post-irradiation by sandwich ELISA. Data are expressed as pg of VEGF × 10⁻²/μg total protein (mean ± S.D.) (grey bars) and as fold increase over unirradiated controls (C) which were set at 1.0 (white bars). **P* = 0.0018 compared with UVB-irradiated HaCaT cells (Student's *t* test). Three independent experiments were performed. Mm (50 nM), cells were treated with 50 nM MTR; UVB (10 mJ/cm²), UVB-irradiated cells; UVB + Mm, HaCaT cells were UVB-irradiated and MTR-treated. S.D., standard deviation.

high frequency of translocations and deletions of the HaCaT cells up to passage numbers around 225 [32].

Antitumour drug MTR reduces the UVB-induced VEGF protein level

To determine molecular mechanisms responsible for the up-regulation of VEGF in response to UVB irradiation and subsequent release of TGFα [31], HaCaT keratinocytes were treated with the antibiotic MTR prior to and after UVB irradiation, and the supernatant was subjected to VEGF₁₆₅ ELISA assays 24 h post-irradiation. MTR inhibits the binding of the transcription factors AP-2 or Sp1 to their GC-rich recognition sites in the VEGF promoter region [40], previously shown to function as TGFα responsive element [15]. An inhibitory effect of MTR on UVB-mediated VEGF protein levels could be detected (Figure 3). A 2.4-fold increase in VEGF protein level after UVB

Figure 4 The UVB-responsive region is localized between -94 and -68 bp with respect to the transcription start site of the VEGF promoter

VEGF promoter deletion constructs were transiently transfected into HaCaT cells and the luciferase activity was determined 12 h post-irradiation and expressed as fold increase over mock-transfected controls which were set at 1.0. (A) Analysis of a sequential series of 5'-deletional VEGF promoter-based luciferase constructs in HaCaT keratinocytes. Values represent mean $\pm$ S.D. of two independent experiments with triplicate transfections per experiment. Nucleotides are numbered from the translation start site; the transcription start site is indicated with an arrow. (B,C) UVB-responsiveness following transient transfection of HaCaT cells with the mutated deletion construct VEGF.9 Δ 3SP-1/AP-2 and the unmutated control constructs VEGF.9 (B), and the mutated full-length promoter construct VEGF.1 Δ 3SP-1/AP-2 and the unmutated control VEGF.1 (C). Values represent the mean $\pm$ S.D. of two independent triplicate transfections. PMA, which confers its effects via the AP-1/AP-2 transcription factors, was tested for its stimulatory effect on the mutated VEGF full-length promoter construct VEGF.1 Δ 3SP-1/AP-2 (C). Mean $\pm$ S.D. of promoter activity was obtained from three independent experiments. The left side of each panel depicts the 5'-flanking region of the human VEGF gene. (B) * $P = 0.57$, # $P = 0.022$ (Student's t test); (C) * $P = 0.69$, # $P = 0.011$, § $P = 0.14$ (Student's t test). S.D., standard deviation.

irradiation at a dose of 10 mJ/cm² compared with the mock-treated HaCaT cells was almost completely abrogated by the application of 50 nM MTR. Treatment of the immortalized cells with MTR alone had no effect on the VEGF level in comparison with mock-treated controls (Figure 3). These data indicated an important role of GC-rich regions in the UVB-triggered induction of VEGF.

An AP-2/Sp1 cluster ($-1133/-1107$ bp) in the 5'-flanking region of the VEGF gene mediates the UVB response

As MTR possesses multiple binding affinities throughout DNA sequences because of its GC-specificity and the observed effects may therefore be due to non-specific binding to other GC-rich

regions, a sequential series of 5'-deletional VEGF promoter-based luciferase reporter gene constructs [10] were used to transiently transfect HaCaT cells. Using these constructs, *cis*-acting GC-rich elements, like AP-2/Sp1 in the VEGF promoter region (Figure 4), were tested for their UVB-responsiveness. In contrast to the promoterless control (pGL-basic), all constructs revealed a significant induction of the luciferase activity compared with the transfected mock-irradiated control cells. After UVB irradiation, even the shortest deletion construct VEGF.9 (-1169 to -988 with respect to the translation initiation site), containing only the combined AP-2/Sp1 cluster in front of the reporter gene, revealed a 1.9-fold induction compared with a 2-fold induction of the full-length promoter construct VEGF.1 (Figure 4A). Consequently, UVB-mediated luciferase activities ranging

Figure 5 MTR significantly attenuated the UVB-stimulated VEGF promoter activity of VEGF in HaCaT cells

Confluent HaCaT cells were transiently transfected with the full-length promoter construct VEGF.1 and incubated with MTR prior to and after UVB irradiation at a dose of 10 mJ/cm². Reporter gene activity was measured 12 h post-irradiation. Values represent the mean $\pm$ S.D. of two independent triplicate transfections and reporter gene assays. Data are expressed as relative light units (RLU; mean $\pm$ S.D.; grey bars) and as fold increase over unirradiated controls (C) which were set at 1.0 (white bars). * P = 0.002 compared with mock-irradiated HaCaT cells (C) (Student's t test). S.D., standard deviation; Mm (50 nM), cells were treated with 50 nM MTR; 10 mJ UVB/cm², UVB-irradiated cells; UVB + Mm, HaCaT cells were UVB-irradiated and MTR-treated.

from 1.9–2.5-fold increases correlate well with the UVB-triggered increase in VEGF₁₆₅ mRNA steady-state [31] and protein levels (Figure 2), suggesting that other components of the UV-regulatory response (i.e. mRNA stability) may not be involved in UVB-dependent VEGF induction. However, further studies are necessary to prove this hypothesis.

The results from the present study provide evidence for a crucial role of the GC-rich AP-2/Sp1 cluster in the UVB induction of VEGF. To further support this conclusion, three out of four Sp1 sites, including the AP-2 site, were deleted by *in vitro* mutagenesis. UVB-responsiveness was tested by transiently transfecting HaCaT cells with the mutated full-length and the shortest deletion promoter constructs, VEGF.1 Δ 3SP-1/AP-2 and VEGF.9 Δ 3SP-1/AP-2 respectively, as well as the control constructs, VEGF.1 and VEGF.9. Complete abrogation of the luciferase activity was observed in HaCaT cells transfected with VEGF.9 Δ 3SP-1/AP-2 (Figure 4B) or VEGF.1 Δ 3SP-1/AP-2 (Figure 4C) following UVB irradiation at a dose of 10 mJ/cm² compared with a 1.9- or 2-fold UVB induction of the luciferase activity in HaCaT cells transfected with the control constructs VEGF.9 and VEGF.1 (Figures 4A and 4B).

In order to exclude the possibility that the introduced mutation in the AP-2/Sp1 cluster interfered with the general recruitment of the transcription initiation complex in the VEGF promoter lacking a TATA sequence, we exposed cells to PMA, a tumour promoter of the phorbol ester class which induces gene expression

by activation of transcription factors like AP-1 and AP-2 [41]. Incubating the cells transfected with the wild-type VEGF.1 construct with PMA resulted in a 1.7-fold increase in the luciferase activity compared with the mock-treated controls. In addition, treatment of the mutated construct VEGF.1 Δ 3SP-1/AP-2 with PMA also led to a significant induction of the luciferase activity in comparison with the UVB-stimulated mutated construct (Figure 4C), confirming that mutation of the AP-2/Sp1 cluster did not promote a general inhibition of gene expression, but specifically abrogated the UVB-induced VEGF promoter activity.

MTR inhibits VEGF promoter activity of transiently transfected HaCaT keratinocytes

To address the question of whether VEGF promoter activity can specifically be inhibited by the drug MTR, HaCaT cells were transiently transfected with the full-length promoter construct VEGF.1. The transfected cells were treated with MTR prior to and after UVB irradiation (Figure 5). The luciferase activity of the VEGF.1 reporter gene construct was determined 12 h after irradiation. UVB irradiation resulted in an 1.5-fold increase in luciferase activity compared with the mock-irradiated transfected HaCaT cells. MTR significantly reduced the promoter activity to the basal level of the mock-irradiated controls. Treatment of the VEGF.1 construct-transfected cells with MTR alone had no effect on the VEGF promoter activity (Figure 5). These data clearly demonstrate that MTR is able to specifically affect the VEGF promoter activity.

Constitutively expressed and UVB-inducible DNA binding activity of nuclear extracts to the –87/–65 bp VEGF promoter sequence

To explore whether specific inducible protein complexes interacted with the DNA sequence whose functional significance had been determined in the promoter activation studies using specific promoter deletion constructs (Figure 4) and the MTR approach (Figure 5), a double-stranded DNA probe corresponding to the –87/–65 bp VEGF promoter sequence containing two adjacent Sp1 transcription factor binding sites and an overlapping AP-2 binding site was utilized in EMSAs (Figure 6). When incubated with nuclear extracts of untreated or sham-irradiated HaCaT cells, constitutive Sp1-dependent binding activity of multiple distinct bands was observed (Figure 6, lane 1). In addition, in lysates of UVB-irradiated cells, an additional prominent DNA binding complex was detected (Figure 6, lane 2), indicating that both constitutively expressed and UVB-induced protein complexes bind to this sequence. To eliminate confusion over constitutive Sp1-dependent binding activity and to focus solely on inducible binding activity, EMSAs were performed in the presence of unlabelled excess Sp1 consensus-oligonucleotide to neutralize constitutive Sp1-dependent complex formation (Figure 6, lanes 3–9). In comparison to sham-irradiated controls (Figure 6, lane 3), UVB radiation markedly induced a distinct Sp1-independent binding complex to the –87/–65 bp VEGF probe (Figure 6, lanes 4, 7 and 8) in a fashion comparable with that seen with UVA (Figure 6, lane 6). As previous studies have identified AP-2 transcription factor in the TGF α - and UVA-induced binding complex [15,29], supershift analysis was performed utilizing antibodies directed against AP-2 protein. Addition of AP-2 antibody indeed resulted in a significant decrease in the intensity of the UVB-inducible complex and in formation of two more slowly migrating complexes (Figure 6, lane 9). These data indicate (i) that the UVB-inducible binding complex is composed

Figure 6 UVB radiation induces AP-2-dependent DNA binding activity to the $-87/-65$ bp VEGF promoter sequence in HaCaT nuclear protein extracts

Representative EMSAs using nuclear extracts of untreated HaCaT cells (lanes 1, 3 and 5), UVB-irradiated (lanes 2, 4, 6, and lanes 7–9) and UVA-exposed cells, either in the absence (lanes 1 and 2) or presence of cold excess double-stranded Sp1-consensus oligonucleotide (lanes 3–9). Formation of specific inducible complexes is indicated on the left by a bold arrow. The DNA sequence of the utilized probe is shown at the top (GC boxes underlined); unlabelled Sp1 consensus oligonucleotides were consistently used at a final concentration of $0.35 \mu\text{M}$; specific AP-2 and irrelevant p65/RelA antibody were added at a concentration of $100 \text{ ng}/\mu\text{l}$. Supershifted complexes are referred to by two small arrows to the right.

of proteins which are almost completely recognized by AP-2 antibody, and therefore (ii) that the UVB-inducible DNA binding complex binds to the $-87/-65$ bp VEGF probe in a Sp1-independent fashion, whereas the constitutively detected DNA-protein complexes bind in a Sp1-dependent manner. An irrelevant antibody directed against p65/NF- κB , however, neither affected the UVB-mediated complex formation nor induced a supershift (Figure 6, lane 8).

DISCUSSION

Under physiological conditions, human cells are exclusively exposed to solar UVB (280–320 nm) and UVA radiation (320–400 nm) because ozone in the stratosphere completely absorbs wavelengths lower than 280 nm. Over the past two decades, continuous decrease of the stratospheric ozone layer was observed [24] which resulted in an increase in UVB intensities reaching human skin and finally in an increase in the incidence of highly vascularized non-melanoma skin cancer [25,42]. UVB radiation induces growth factors and cytokines, such as basic fibroblast growth factor [42], TNF α [43], IL-1, and IL-6 [44]. These are also involved in the induction of VEGF, the major growth factor for angiogenesis, the growth of new capillaries from pre-existing blood vessels which is essential for cancers to grow beyond minimal size [45,46]. Apart from the known properties of VEGF as an endothelial cell-specific mitogen and as a vascular permeability factor, it has recently been shown that VEGF can provoke a more malignant phenotype of epithelial tumour cells *in vivo*, suggesting a further significant role of

VEGF in facilitating tumour progression by enhancing differentiation and enhanced tumour infiltration [47]. Thus, premalignant cell lines such as HaCaT are a valuable tool to study the mechanisms by which premalignant phenotypes switch to malignant phenotypes.

Even though many studies have dealt with the effect of VEGF and some novel VEGFs on angiogenesis [46,48,49], the molecular mechanisms underlying UVA-, and especially UVB-, mediated VEGF induction have been poorly understood. Recently, Gille et al. [29] convincingly demonstrated that UVA rapidly induced VEGF mRNA in a fashion comparable with that seen in response to TGF α [15].

As UVB radiation is a pivotal initiator of photocarcinogenesis and inducer of VEGF [30,31], research on the molecular mechanisms underlying UVB-modulated VEGF expression is a matter of increasing interest and relevance since it may provide ultimate clues for better understanding of angiogenesis in pathological states and for a rational design of anti-angiogenic therapies. Recently, we reported on a biphasic UVB-dependent induction of VEGF in the human epidermal cell line HaCaT [31]. We extended these investigations in the present study and examined the molecular mechanisms leading to the second VEGF peak 12–24 h upon UVB irradiation at relevant doses which can be easily acquired on a clear day at a latitude between 48 to 56 degrees north [50].

In order to determine the sequence requirements for UVB-mediated VEGF induction, we utilized 5'-deletional VEGF promoter-based reporter gene constructs and mutations of these constructs. Since UVB has been shown to release Fe(II) from iron-binding proteins [51], and also may interfere with the concentration and conformation of metalloproteins which have been postulated to operate as oxygen sensors in the regulation of hypoxia-induced gene expression [52], we initially hypothesized that the UVB-response recognition sequence may be contained in the hypoxia response element of the VEGF promoter. However, two independent studies have located a hypoxia response element between nucleotides -985 and -951 [53] and -1180 and -887 [38], which is distinctly different from the UVB-response element identified in the present study. This element is located between -1126 and -1104 bp with respect to the translation initiation site, and -87 to -65 bp with respect to the transcription start site, containing two Sp1 sites and an AP-2 site which overlaps a Sp1 site. This overlapping AP-2/Sp1 cluster ($-78/-68$ bp) was found to be critical for both constitutive and UVB-induced transcriptional activity. Our data correlate with earlier findings that AP-2-dependent DNA binding and transactivation are required for VEGF gene expression in response to TGF α , as well as UVA radiation, using identical AP-2/Sp1 recognition sites in close proximity to the transcription start site [29]. Although AP-2 transcription factor was validated in the present study to be an essential mediator of VEGF induction, it does not represent a general or exclusive mechanism in VEGF induction. This assumption is supported by recent studies [54] showing that different nuclear transcription factors, e.g. AP-1 and HIF-1 (hypoxia inducible factor-1), are responsible for transactivation of the VEGF gene and dependent on distinct extracellular stimuli.

In addition to VEGF, the results from the present study regarding the importance and function of AP-2 recognition sites which overlap with Sp1 sites has been described for other genes, e.g. for induction of t-PA [55] and for activity of the intercellular adhesion molecule-1 (ICAM-1) promoter [56]. So far, to our knowledge, VEGF is the first gene which has been demonstrated to be functionally activated via UVB-initiated AP-2/Sp1 transactivation. Together with earlier published results [15,29,56] our data strengthen the concept of a more general importance of AP-

2 in UV radiation-mediated responses of keratinocytes, which is supported by the presence of putative functional AP-2 binding sites in other UV-regulated genes [57]. In previously published reports, mainly the UVB-triggered transactivation of transcription factor AP-1 was the focus of interest and therefore AP-1 has often been thought to be the master switch for UVB-initiated gene induction [58,59].

Furthermore, the data from the present study indicate that, at least for VEGF expression, UVB and UVA irradiation may use the same signalling pathway, finally leading to binding of the corresponding transcription factors to the AP-2/Sp1 cluster in the promoter region in close proximity to the transcription start site. This hypothesis can be supported by the findings of Blandschun et al. [31] and Gille et al. [29] who identified TGF α as a pivotal mediator for both the UVB and UVA response in terms of VEGF expression. This finding is not completely in agreement with the concept that mechanisms by which UVB irradiation induces transcriptional activation of human genes differ from those induced by UVC or UVA irradiation. These differences are thought to be due to the finding that biological effects induced by short and long wavelength UV radiation involve different chromophores leading to different photobiological effects [33,60,61].

In conclusion, our findings underline an essential role of AP-2/Sp1 recognition sites in UVB-mediated VEGF expression by the keratinocyte-derived cell line HaCaT serving as a model for a transformed pre-malignant epithelial cell line. Understanding the exact UVB-triggered molecular mechanisms which result in the up-regulation of the angiogenic VEGF may be of considerable interest for the development of novel sunscreens and other anti-angiogenic strategies to prevent neo-vascularization in pathological disorders such as cutaneous photoaging and skin cancer.

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REFERENCES

- Scharfetter-Kochanek, K., Wlaschek, M., Brenneisen, P., Schauen, M., Blandschun, R. and Wenk, J. (1997) UV-induced reactive oxygen species in photocarcinogenesis and photoaging. *Biol. Chem.* **378**, 1247–1257.
- Brash, D. E., Rudolph, J. A., Simon, J. A., Lin, A., McKenna, G. J., Baden, H. P., Halperin, A. J. and Ponten, J. (1991) A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10124–10128.
- Soffen, G. A. and Blum, H. F. (1961) Quantitative measurements of changes in mouse skin following a single dose of ultraviolet light. *J. Cell Compar. Physiol.* **58**, 81–96.
- Hanahan, D. and Folkman, J. (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86**, 353–364.
- Skobe, M., Rockwell, P., Goldstein, N., Vosseler, S. and Fusenig, N. E. (1997) Halting angiogenesis suppresses carcinoma cell invasion. *Nature Med.* **3**, 1222–1227.
- Kashani-Sabet, M., Sagebiel, R. W., Ferreira, C. M., Nosrati, M. and Miller, J. R. 3rd (2001) Vascular involvement in the prognosis of primary cutaneous melanoma. *Arch. Dermatol.* **137**, 1169–1173.
- Keck, P. J., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feder, J. and Connolly, D. T. (1989) Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* **246**, 1309–1312.
- Claffey, K. P. and Robinson, G. S. (1996) Regulation of VEGF/VPF expression in tumor cells: consequences for tumor growth and metastasis. *Cancer and Metastasis Rev.* **15**, 165–176.
- Tischer, E., Mitchell, R., Hartman, T., Silva, M., Gospodarowicz, D., Fiddes, J. C. and Abraham, J. A. (1991) The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J. Biol. Chem.* **266**, 11947–11954.
- Damert, A., Ikeda, E. and Risau, W. (1997) Activator-protein-1 binding potentiates the hypoxia-inducible factor-1-mediated hypoxia-induced transcriptional activation of vascular endothelial growth factor expression in L6 glioma cells. *Biochem. J.* **327**, 419–423.
- Valter, M. M., Wiestler, O. D. and Pietsche, T. (1999) Differential control of VEGF synthesis and secretion in human glioma cells by IL-1 and EGF. *Int. J. Dev. Neurosci.* **17**, 565–577.
- Cohen, T., Nahari, D., Cerem, L. W., Neufeld, G. and Levi, B. Z. (1996) Interleukin 6 induces the expression of vascular endothelial growth factor. *J. Biol. Chem.* **271**, 736–741.
- Bottomly, M. J., Webb, N. J., Watson, C. J., Holt, P. J., Freemont, A. J. and Brenchley, P. E. (1999) Peripheral blood mononuclear cells from patients with rheumatoid arthritis spontaneously secrete vascular endothelial growth factor (VEGF): specific up-regulation by tumor necrosis factor- α (TNF α) in synovial fluid. *Clin. Exp. Immunol.* **117**, 171–176.
- Pertovaara, L., Kaipainen, A., Mustonen, T., Orpana, A., Ferrara, N., Saksela, O. and Alitalo, K. (1994) Vascular endothelial growth factor is induced in response to transforming growth factor- β in fibroblastic and epithelial cells. *J. Biol. Chem.* **269**, 6271–6274.
- Gille, J., Swerlick, R. A. and Caughman, W. (1997) Transforming growth factor- α -induced transcriptional activation of the vascular permeability factor (VPF/VEGF) gene requires AP-2-dependent DNA binding and transactivation. *EMBO J.* **16**, 750–759.
- Matsumoto, T. and Claesson-Welsh, L. (2001) VEGF receptor signal transduction. *Sci. STKE* **112**, RE21.
- Ferrara, N. and Davis-Smyth, T. (1997) The biology of vascular endothelial growth factor. *Endocr. Rev.* **18**, 4–25.
- Brown, L. F., Yeo, K. T., Berse, B., Yeo, T. K., Senger, D. R., Dvorak, H. F. and van de Water, L. (1992) Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing. *J. Exp. Med.* **176**, 1375–1379.
- Detmar, M., Brown, L., Claffey, K., Yeo, K. T., Kocher, O., Jackman, R. W., Berse, B. and Dvorak, H. F. (1994) Overexpression of vascular permeability factor and its receptors in psoriasis. *J. Exp. Med.* **180**, 1141–1146.
- Detmar, M. (1996) Molecular regulation of angiogenesis in the skin. *J. Invest. Dermatol.* **106**, 207–208.
- Blandschun, R., Sunderkötter, C., Brenneisen, P., Hinrichs, R., Peters, T., Schneider, L., Razi-Wolf, Z., Hunzelmann, N. and Scharfetter-Kochanek, K. (2002) Vascular endothelial growth factor causally contributes to the angiogenic response upon ultraviolet B irradiation *in vivo*. *Br. J. Dermatol.* **146**, 581–587.
- Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S. and Ferrara, N. (1993) Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth *in vivo*. *Nature (London)* **362**, 841–844.
- Lloyd, S. A. (1993) Stratospheric ozone depletion. *Lancet* **342**, 1156–1160.
- De Fazio, E. C. (2000) Stratospheric ozone depletion: UVB “effects”, the neglected aspect. *Int. J. Circumpolar Health* **59**, 2–3.
- Slaper, H., Velder, G. J. M., Daniel, J. S., de Gruij, F. R. and van der Leun, J. C. (1996) Estimates of ozone depletion and skin cancer incidence to examine the Vienna Convention achievements. *Nature (London)* **384**, 256–258.
- Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A. and Fusenig, N. E. (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* **106**, 761–771.
- Fusenig, N. E. and Boukamp, P. (1998) Multiple stages and genetic alterations in immortalization, malignant transformation, and tumor progression of human skin keratinocytes. *Mol. Carcinog.* **23**, 144–158.
- Mildner, M., Weninger, W., Trautinger, F., Ban, J. and Tschachler, E. (1999) UVA and UVB radiation differentially regulate vascular endothelial growth factor expression in keratinocyte-derived cell lines and in human keratinocytes. *Photochem. Photobiol.* **70**, 674–679.
- Gille, J., Reisinger, K., Asbe-Vollkopf, A., Hardt-Weinelt, K. and Kaufmann, R. (2000) Ultraviolet-A-induced transactivation of the vascular endothelial growth factor gene in HaCaT keratinocytes is conveyed by activator protein-2 transcription factor. *J. Invest. Dermatol.* **115**, 30–36.
- Brauchle, M., Funk, J. O., Kind, P. and Werner, S. (1996) Ultraviolet B and H₂O₂ are potent inducers of vascular endothelial growth factor expression in cultured keratinocytes. *J. Biol. Chem.* **271**, 21793–21797.
- Blandschun, R., Brenneisen, P., Wlaschek, M., Meewes, C. and Scharfetter-Kochanek, K. (2000) 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 . *FEBS Lett.* **474**, 195–200.
- Boukamp, P., Popp, S., Altmeyer, S., Hulsen, A., Fasching, C., Cremer, T. and Fusenig, N. E. (1997) Sustained nontumorigenic phenotype correlates with a largely stable chromosome content during long-term culture of the human keratinocyte line HaCaT. *Genes Chromosomes Cancer* **19**, 201–214.

- 33 Brenneisen, P., Wenk, J., Wlaschek, M., Krieg, T. and Scharffetter-Kochanek, K. (2000) Activation of p70 ribosomal S6 protein kinase is an essential step in the DNA damage-dependent signaling pathway responsible for the ultraviolet B-mediated increase in interstitial collagenase (MMP-1) and stromelysin-1 (MMP-3) protein levels in human dermal fibroblasts. *J. Biol. Chem.* **275**, 4336–4344
- 34 Herzinger, T., Wolf, D. A., Eick, D. and Kind, P. (1995) The pRb-related protein p130 is a possible effector of transforming growth factor β 1 induced cell cycle arrest in keratinocytes. *Oncogene* **11**, 2151–2156
- 35 Prado, L., Fernandez, E., Weissbach, U., Blanco, G., Quiros, L. M., Brana, A. F., Mendez, C., Rohr, J. and Salas, J. A. (1999) Oxidative cleavage of premithramycin B is one of the last steps in the biosynthesis of the antitumor drug mithramycin. *Chem. Biol.* **6**, 19–30
- 36 Green, L. M., Reade, J. L. and Ware, C. F. (1984) Rapid colorimetric assay for cell viability: application to the quantitation of cytotoxic and growth inhibitory lymphokines. *J. Immunol. Methods* **70**, 257–268
- 37 Houck, K. A., Ferrara, N., Winer, J., Cachianes, G., Li, B. and Leung, D. W. (1991) The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol. Endocrinol.* **5**, 1806–1814
- 38 Ikeda, E., Achen, M. G., Breier, G. and Risau, W. (1995) Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells. *J. Biol. Chem.* **270**, 19761–19766
- 39 Pogulis, R. J., Vallejo, A. N. and Pease, L. R. (1996) *In vitro* recombination and mutagenesis by overlap extension PCR. *Methods Mol. Biol.* **57**, 167–176
- 40 Snyder, R. C., Ray, R., Blume, S. and Miller, D. M. (1991) Mithramycin blocks transcriptional initiation of the c-myc P1 and P2 promoters. *Biochemistry* **30**, 4290–4297
- 41 Kolch, W., Martiny-Baron, G., Kieser, A. and Marme, D. (1995) Regulation of the expression of the VEGF/VPS and its receptors: role in tumor angiogenesis. *Breast Cancer Res. Treat.* **36**, 139–155
- 42 Bielenberg, D. R., Bucana, C. D., Sanchez, R., Donawho, C. K., Kripke, M. L. and Fidler, I. J. (1998) Molecular regulation of UVB-induced cutaneous angiogenesis. *J. Invest. Dermatol.* **112**, 802–809
- 43 Kibitel, J., Hejmadi, V., Alas, L., O'Connor, A., Sutherland, B. M. and Yarosh, D. (1998) UV-DNA damage in mouse and human cells induces the expression of tumor necrosis factor α . *Photochem. Photobiol.* **67**, 541–546
- 44 Eberlein-König, B., Jäger, C. and Przybilla, B. (1998) Ultraviolet B radiation-induced production of interleukin-1 α and interleukin-6 in a human squamous carcinoma cell line is wavelength-dependent and can be inhibited by pharmacological agents. *Br. J. Dermatol.* **139**, 415–421
- 45 Folkman, J. (1992) The role of angiogenesis in tumor growth. *Sem. Cancer Biol.* **3**, 65–71
- 46 Ferrara, N. (1999) Vascular endothelial growth factor: molecular and biological aspects. *Curr. Top. Microbiol. Immunol.* **237**, 1–30
- 47 Detmar, M., Velasco, P., Richard, L., Claffey, K. P., Streit, M., Riccardi, L., Skobe, M. and Brown, L. F. (2000) Expression of vascular endothelial growth factor induces an invasive phenotype in human squamous cell carcinomas. *Am. J. Pathol.* **156**, 159–167
- 48 Risau, W. (1997) Mechanisms of angiogenesis. *Nature (London)* **386**, 671–674
- 49 Yancopoulos, G. D., Davis, S., Gale, N. W., Rudge, J. S., Wiegand, S. J. and Holash, J. (2000) Vascular-specific growth factors and blood vessel formation. *Nature (London)* **407**, 242–248
- 50 Jeanmougin, M. and Civate, J. (1987) Benign summer light eruption: the most common photodermatosis. *J. Am. Acad. Dermatol.* **17**, 690–691
- 51 Biemond, P., Swaak, A. J., van Eijk, H. G. and Koster, J. F. (1988) Superoxide dependent iron release from ferritin in inflammatory diseases. *Free Radic. Biol. Med.* **4**, 185–198
- 52 Goldberg, M. A. and Schneider, T. J. (1994) Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. *J. Biol. Chem.* **269**, 4355–4359
- 53 Liu, Y., Cox, S. R., Morita, T. and Kourembanas, S. (1995) Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer. *Circ. Res.* **77**, 638–643
- 54 Shih, S. C. and Claffey, K. P. (2001) Role of AP-1 and HIF-1 transcription factors in TGF- β activation of VEGF expression. *Growth Factors* **19**, 19–34
- 55 Costa, M., Shen, Y., Maurer, F. and Medcalf, R. L. (1998) Transcriptional regulation of the tissue-type plasminogen-activator gene in human endothelial cells: identification of nuclear factors that recognise functional elements in the tissue-type plasminogen-activator gene promoter. *Eur. J. Biochem.* **258**, 123–131
- 56 Grether-Beck, S., Olazola-Horn, S., Schmitt, H., Grewe, M., Jahnke, A., Johnson, J. P., Briviba, K., Sies, H. and Krutmann, J. (1996) Activation of transcription factor AP-2 mediates UVA radiation- and singlet oxygen-induced expression of the human intercellular adhesion molecule-1 gene. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 14586–14591
- 57 Grether-Beck, S., Buettner, R. and Krutmann, J. (1997) Ultraviolet A-radiation-induced expression of human genes: molecular and photobiological mechanisms. *Biol. Chem.* **378**, 1231–1236
- 58 Garzyn, M. and Degreef, H. (1997) Suppression of UVB-induced c-fos and c-jun expression in human keratinocytes by N-acetylcysteine. *J. Photochem. Photobiol. B: Biology* **37**, 125–130
- 59 Karin, M., Liu, Z.-G. and Zandi, E. (1997) AP-1 function and regulation. *Curr. Opin. Cell Biol.* **9**, 240–246
- 60 Wlaschek, M., Heinen, G., Poswig, A., Schwarz, A., Krieg, T. and Scharffetter-Kochanek, K. (1994) UVA-induced autocrine stimulation of fibroblast-derived collagenase/MMP-1 by interrelated loops of interleukin-1 and interleukin-6. *Photochem. Photobiol.* **59**, 550–556
- 61 Wang, Y., Rosenstein, B., Goldwyn, S., Zhang, X., Lebwohl, M. and Wei, H. (1998) Differential regulation of P53 and Bcl-2 expression by ultraviolet A and B. *J. Invest. Dermatol.* **111**, 380–384

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