Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is strongly expressed by epidermal keratinocytes during wound healing, in psoriasis, and in bullous diseases such as erythema multiforme and bullous pemphigoid. All of these disorders are characterized by increased microvascular permeability and angiogenesis. Since the development of erythema as a result of hyperpermeable blood vessels is a common feature after excess sun exposure, we speculated about an up-regulation of VEGF expression by ultraviolet (UV) light. To test this hypothesis, we analyzed the effect of UVB irradiation on VEGF expression in cultured keratinocytes. Thereby we found a large increase in VEGF mRNA and protein levels upon irradiation of quiescent keratinocytes with sublethal doses of UVB. Although H$_2$O$_2$ was also a potent inducer of VEGF expression, the effect of UVB irradiation is unlikely to be mediated by reactive oxygen species as determined by the use of antioxidants. Further experiments revealed that the UVB-induced overexpression of VEGF is dependent on de novo protein synthesis and might occur via release of soluble mediators, which subsequently turn on VEGF expression. In summary, our results suggest a novel role of VEGF in the induction of erythema after excess sun exposure.

**MATERIALS AND METHODS**

**Cell Culture**—The human keratinocyte cell line HaCaT (26) was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml). For experiments with H$_2$O$_2$, keratinocytes were grown in RPMI 1640 medium supplemented with the same components as above. This medium does not contain iron salts, which are known to promote the decomposition of H$_2$O$_2$. In both cases, cells were grown to confluence without changing the medium and rendered quiescent by a 16-h incubation in serum-free medium. Cells were then incubated for varying periods in serum-free medium containing H$_2$O$_2$, pyrrolidine dithiocarbamate (PDTC), cycloheximide, or conditioned medium from UVB-treated cells. Dulbecco’s modified Eagle’s medium, RPMI 1640, and fetal bovine serum were purchased from Life Technologies, Inc. PDTC, cycloheximide, and H$_2$O$_2$ were from Sigma.
UVB Irradiation of Cells—For UVB treatment, confluent and quiescent cells were washed twice with phosphate-buffered saline, irradiated while under a thin film of phosphate-buffered saline, and replenished with their own medium thereafter. The UVB source was a parallel bank of three TL20/12 fluorescent tubes (Philips, Hamburg) emitting a continuous spectrum between 280 and 320 nm with a peak emission at 312 nm. Fluence rate at the site of cell irradiation was 0.86 milliwatts/cm² as measured with a Centra radiometer (Osram, Munich). The UVB doses employed ranged from 5 to 20 mJ/cm² (exposure time: 7–26 s) and were sublethal for the cells (data not shown). These doses are a realistic representation of the irradiation reaching basal keratinocytes in vivo (27). Aliquots of cells were harvested before and at different time points after UVB irradiation and used for RNA isolation.

RNA Isolation and RNase Protection Assay—RNA isolation was performed as described (28). RNase protection assays were carried out as published recently (29). Briefly, a 159-base pair fragment corresponding to nucleotides 339–498 of the human VEGF121 cDNA (30) was cloned into the transcription vector pBluescript KSII(+) (Stratagene) and linearized at the 5′-end. An antisense transcript was synthesized in vitro using T3 RNA polymerase and [³²P]JUTP (800 Ci/mmol, Amerham). 20 μg of total cellular RNA was hybridized at 42°C overnight with 100,000 cpm of the labeled antisense transcript. Hybrids were digested with RNases A and T1 for 1 h at 30°C. Protected fragments were separated on 5% acrylamide, 8 M urea gels and analyzed by autoradiography. The increase in VEGF mRNA levels was quantitated by Phosphorimager analysis (FUJI BAS 1000, Fuji).

Western Blot Analysis of VEGF Protein—5 ml of serum-free Dulbecco’s modified Eagle’s medium/10-cm Petri dish was conditioned by UVB-irradiated quiescent HaCaT cells. 8 and 24 h after irradiation the conditioned medium from three Petri dishes was harvested and centrifuged to remove cell debris. Heparin-binding proteins were precipitated from the supernatant with 100 μl of heparin-Sepharose (1:1 slurry; Pharmacia) overnight at 4°C. Heparin-Sepharose beads were precipitated by centrifugation and washed three times with 50 mM Tris-HCl, pH 7.4, 300 mM NaCl. Heparin-Sepharose-bound proteins were extracted by a 5-min incubation in Laemmli sample buffer at 95°C and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After transfer to nitrocellulose membranes, VEGF proteins were detected using a polyclonal antiserum directed against the amino terminus of VEGF (Santa Cruz Biotechnology) and an alkaline phosphatase detection system (Promega). Conditioned medium from nontreated cells was used as a negative control, whereas a cell lysate from COS cells transiently transfected with a VEGF cDNA was used as a positive control.

RESULTS

Increased VEGF mRNA and Protein Levels after UVB Irradiation in the Human Keratinocyte Cell Line HaCaT—To investigate the role of sunlight on VEGF expression in the skin, we studied the effect of UVB on VEGF expression in the human keratinocyte cell line HaCaT. For this purpose, cells were irradiated with physiologically relevant doses of UVB and harvested at different time points after UVB treatment. As shown in Fig. 1A, low levels of VEGF mRNA were detected in quiescent, nontreated keratinocytes. Within 5 h after UVB irradiation (10 and 20 mJ/cm²), a large induction of VEGF mRNA expression was observed with highest levels 8 h after exposure to 20 mJ of UVB/cm² (Fig. 1, A and B). At this time point, VEGF mRNA levels were 11-fold higher compared with the basal level. This effect was long lasting, and 24 h after exposure of the cells to UVB, VEGF mRNA levels were still elevated (2-3-fold).

Four different VEGF proteins have recently been identified in vitro which arise from differential splicing at the 3′-end of VEGF primary transcripts (12, 13). The hybridization probe that we used for RNase protection assays corresponds to the 3′-end of the shortest form of VEGF (VEGF121) and thus enabled us to distinguish among the different isoforms of this growth factor (Fig. 1C). A protected fragment corresponding to the complete coding sequence of the hybridization probe is generated by mRNA encoding VEGF121 (upper band in Fig. 1A), whereas two shorter protected fragments are generated by mRNAs encoding longer variants of VEGF (lower bands in Fig. 1A). mRNAs encoding VEGF121 and other splice variants were induced to a similar extent (Fig. 1A).

To analyze whether the observed increase in VEGF mRNA levels correlates with the production of immunoreactive VEGF protein, conditioned medium from UVB-irradiated and nonirradiated HaCaT cells was analyzed for the presence of VEGF protein. Because of the kinetics of the VEGF induction at the mRNA level, conditioned medium was harvested 8 and 24 h after irradiation, and VEGF protein was enriched by its capacity to bind to heparin-Sepharose. The presence of VEGF protein in the conditioned medium was detected by immunoblotting using a polyclonal antiserum directed against the amino terminus of VEGF, which is identical in all isoforms. As shown in Fig. 2, two VEGF proteins with estimated molecular masses of 17 and 19 kDa were detected in the conditioned medium of UVB-treated cells. The size of these proteins correlates with the expected sizes of the VEGF gene products. A VEGF specific
Cycloheximide on UVB- and H2O2-induced VEGF expression—investigated the effect of the protein synthesis inhibitor on VEGF expression in HaCaT cells. The conditioned medium of three 10-cm Petri dishes was harvested 8 and 24 h after treatment. As a control, conditioned medium from nontreated cells was collected. Heparin-binding proteins were enriched by their capacity to bind to heparin-Sepharose beads and analyzed by immunoblotting for the presence of VEGF protein. Two VEGF proteins of approximately 17 and 19 kDa (indicated with arrows) were detected 8 and 24 h after UVB irradiation. Cell lysate of COS cells transiently transfected with a VEGF cDNA was used as a positive control.

Dependent on de Novo Protein Synthesis—UVB irradiation of cells has been shown to increase intracellular levels of reactive oxygen species (31). Oxygen Radicals—UV irradiation correlates with accumulation of VEGF protein in the medium.

**Induction of VEGF Expression by UVB Is Not Mediated by Reactive Oxygen Species.** Quiescent HaCaT cells were irradiated with 20 mJ/cm² of UVB, and the conditioned medium of three 10-cm Petri dishes was harvested 8 and 24 h after treatment. As a control, conditioned medium from nontreated cells was collected. Heparin-binding proteins were enriched by their capacity to bind to heparin-Sepharose beads and analyzed by immunoblotting for the presence of VEGF protein. Two VEGF proteins of approximately 17 and 19 kDa (indicated with arrows) were detected 8 and 24 h after UVB irradiation. Cell lysate of COS cells transiently transfected with a VEGF cDNA was used as a positive control.

**Fig. 2. Induction of VEGF mRNA expression by UVB irradiation correlates with accumulation of VEGF protein in the conditioned medium.** Quiescent HaCaT cells were irradiated with 20 mJ/cm² of UVB, and the conditioned medium of three 10-cm Petri dishes was harvested 8 and 24 h after treatment. As a control, conditioned medium from nontreated cells was collected. Heparin-binding proteins were enriched by their capacity to bind to heparin-Sepharose beads and analyzed by immunoblotting for the presence of VEGF protein. Two VEGF proteins of approximately 17 and 19 kDa (indicated with arrows) were detected 8 and 24 h after UVB irradiation. Cell lysate of COS cells transiently transfected with a VEGF cDNA was used as a positive control.

**Fig. 3. Induction of VEGF mRNA expression by UVB is not mediated by reactive oxygen species.** Panel A, keratinocytes were rendered quiescent by serum starvation and treated with 1 mM H2O2 for 5 and 8 h. Samples of 20 μg of total cellular RNA were analyzed for VEGF mRNA expression by RNase protection assay. 50 μg of RNA was used as a negative control. 1,000 cpm of the hybridization probe was used as a size marker. In the same experiment, the cells were treated for 1 h with PDTC (20 μM) before the addition of H2O2 (1 mM) or treated exclusively with PDTC (panel B). Panel C, quiescent keratinocytes were irradiated with 20 mJ/cm² UVB with or without pretreatment with PDTC (20 μM). Total cellular RNA was harvested at different time points as indicated, and 20 μg of RNA was analyzed for VEGF mRNA expression by RNase protection assay.

**DISCUSSION**

Solar radiation causes a variety of biological effects in the skin. Whereas low doses of UV radiation can be useful due to...
the photosynthesis of vitamin D, larger doses cause sunburn and, when exposure is chronic, can promote tumor formation. Sunburn is characterized by erythema and edema as a result of increased vascular permeability. The mechanisms that underlie these effects have not been elucidated fully. Since erythema is particularly induced by UVB irradiation that only penetrates into the epidermis (33), keratinocyte-derived soluble mediators that diffuse to the dermis are likely to mediate this effect. This diffusion theory is supported by the lag phase between UVB exposure and the appearance of visible erythema and by the increase in the latent period for visible erythema after cooling of the skin (34). The soluble mediators that induce increased vascular permeability have only partially been characterized. Previous studies have suggested an important role of prostaglandins in this process. However, the later stages of erythema cannot be explained fully by the presence of these factors (for review, see Ref. 33). Therefore, additional mediators might contribute to the effect.

One of the most potent vascular permeability factors is VEGF, which is overexpressed in a wide variety of physiological and pathological conditions associated with increased vascular permeability, such as cancer and wound healing and also in erythema seen in several blistering diseases (1, 9, 21). The results described in the present study suggest an additional role of VEGF in UVB-induced erythema. UVB irradiation of cultured keratinocytes resulted in a strong increase in VEGF mRNA and protein levels. These elevated levels might either be due to transcriptional activation of the VEGF gene and/or to increased mRNA stability since both mechanisms have been shown to contribute to the regulation of VEGF mRNA levels (35).

The UVB dose and spectrum used in this study were well comparable to the UVB light reaching the human skin upon sun exposure, suggesting that a similar increase in VEGF expression might occur in vivo. Thus, in addition to prostaglandins, VEGF might contribute further to the induction of erythema by UVB irradiation. This hypothesis is supported by a recent study that demonstrated a synergistic effect of prostaglandin E2 and VEGF in a quantitative model of local plasma leakage in rabbit skin (36), and a similar synergistic effect might occur in human skin after sun exposure.

The effects of UV irradiation are frequently mediated by reactive oxygen species, and increased levels of hydrogen peroxide, hydroxyl radicals, superoxide, and organic hydroperoxides are found in many different cell types following UV exposure (31, 33). These reactive oxygen species are potent modulators of transcription factor activity (37, 38), resulting in significant changes in gene expression. Therefore we speculated about a role of these molecules in the induction of VEGF expression seen after UVB exposure. Indeed, treatment of keratinocytes with H2O2 caused a strong increase in VEGF expression which was blocked by antioxidants. By contrast, antioxidants had no effect on the UVB-induced VEGF expression, suggesting that UVB irradiation and H2O2 induce expression of this gene via different mechanisms. The induction of VEGF expression by UVB in the presence of antioxidants is in agreement with recent results that demonstrated the involvement of reactive oxygen intermediates in the UVA but not in the UVB response (39).

A series of previous studies suggested an important role of tyrosine phosphorylation but not of protein kinase C activation in the induction of gene expression by UVC and UVB (40–44),
and preliminary studies of our laboratory suggest that similar mechanisms might be responsible for the increased expression of VEGF after UVB irradiation. Thus, pretreatment of the cells with protein tyrosine kinase inhibitors blocked the UVB response, whereas protein kinase C inhibitors had no effect.

Interestingly, induction of VEGF expression in keratinocytes by H₂O₂ and various growth factors and cytokines occurred much more rapidly compared with the induction seen after UVB exposure (32 and data not shown). This finding suggested that indirect mechanisms might be responsible for the increased expression and preliminary studies of our laboratory suggest that similar mechanisms might be responsible for the increased expression of VEGF after UVB irradiation. Thus, pretreatment of the cells with protein tyrosine kinase inhibitors blocked the UVB response, whereas protein kinase C inhibitors had no effect. By H₂O₂ and various growth factors and cytokines, as well as a combined action of these factors, might be responsible for the increased expression and preliminary studies of our laboratory suggest that similar mechanisms might be responsible for the increased expression of VEGF after UVB irradiation. Thus, pretreatment of the cells with protein tyrosine kinase inhibitors blocked the UVB response, whereas protein kinase C inhibitors had no effect.

Acknowledgments—We thank Dr. Peter Hans Hofschneider for helpful suggestions and support, Frédérique Torterotot for excellent technical assistance, and Dr. H. W. for the human VEGF cDNA.

REFERENCES
1. Dvorak, H. F., Brown, L. F., Detmar, M., and Dvorak, A. M. (1995) Am. J. Pathol. 146, 1029–1039
2. Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S., and Dvorak, H. F. (1983) Science 219, 983–985
3. Senger, D. R., Connolly, D., Perruzzi, C. A., Alsup, D., Nelson, R., Leimgruber, R., Fedor, J., and Dvorak, H. F. (1987) Fed. Proc. 46, 2102
4. Connolly, D. T., Olander, J. V., Heuvelman, D., Nelson, R., Monsell, R., Siegel, N., Haymore, B. L., Leimgruber, H., and Fedor, J. (1989) J. Biol. Chem. 264, 20017–20024
5. Senger, D. R., Connolly, D., Van De Water, L., Fedor, J., and Dvorak, H. F. (1990) Cancer Res. 50, 1774–1778
6. Ferrara, N., and Henzel, W. J. (1989) Biochem. Biophys. Res. Commun. 161, 851–858
7. Gospodarowicz, D., Abraham, J. A., and Schilling, J. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 7311–7315
8. Conn, G., Soderman, D. D., Schaeffer, M. T., Wire, M., Hatcher, V. B., and Thomas, K. A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1323–1327
9. Brown, P. J., Feo, K. T., Bese, B., Yeo, T.-K., and Dvorak, H. F. (1992) Biochem. Biophys. Res. Commun. 186, 1141–1146
10. Keck, P. J., Hauser, S. D., Kriwil, G., Sanzo, K., Warren, T., Feder, J., and Connolly, D. T. (1989) Science 243, 1309–1312
11. Tischer, E., Mitchell, R. I., Trefzer, U., and Dvorak, H. F. (1994) J. Exp. Med. 180, 1187–1194
12. Hock, K. A., Ferrara, N., Winer, J., and Cachianes, G. (1990) Mol. Endocrinol. 5, 1806–1814
13. Breier, G., Albrecht, U., Sterrer, S., and Risau, W. (1992) Development 114, 521–532
14. Ferrara, N., Stumpf, W., and Risau, W. (1995) Development 121, 2102–2108
15. Dvorak, H. F. (1983) J. Biol. Chem. 258, 1029–1039
16. Van De Water, L. (1992) Biochem. Biophys. Res. Commun. 186, 67S–70S
17. Shima, D. T., Deutsch, U., and D’Amore, P. A. (1995) FEBS Lett. 370, 203–208
18. Bhatia, R., and Dvorak, H. F. (1987) J. Cell Biol. 104, 87–97
19. Shima, D. T., Deutsch, U., and D’Amore, P. A. (1995) FEBS Lett. 370, 203–208
20. Bhatia, R., and Dvorak, H. F. (1987) J. Cell Biol. 104, 87–97
Ultraviolet B and \( \text{H}_2\text{O}_2 \) Are Potent Inducers of Vascular Endothelial Growth Factor Expression in Cultured Keratinocytes
Maria Brauchle, Jens Oliver Funk, Peter Kind and Sabine Werner

*J. Biol. Chem.* 1996, 271:21793-21797.
doi: 10.1074/jbc.271.36.21793

Access the most updated version of this article at [http://www.jbc.org/content/271/36/21793](http://www.jbc.org/content/271/36/21793)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 44 references, 19 of which can be accessed free at [http://www.jbc.org/content/271/36/21793.full.html#ref-list-1](http://www.jbc.org/content/271/36/21793.full.html#ref-list-1)