Low Concentrations of Curcumin Induce Growth Arrest and Apoptosis in Skin Keratinocytes Only in Combination with UVA or Visible Light

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It is well known that curcumin, a dietary pigment from the plant *Curcuma longa*, inhibits cell proliferation and induces apoptosis in different cell lines at concentrations ranging from 10 to 150 μM (3.7–55 μg/ml). In this study, we show that curcumin at low concentrations (0.2–1 μg/ml) also has an antiproliferative effect when applied in combination with UVA or visible light. We demonstrate that such a treatment induces apoptosis in human skin keratinocytes represented by the increase of fragmented cell nuclei, release of cytochrome *c* from mitochondria, activation of caspases-9 and -8, and inhibition of NF-κB activity. Furthermore, inhibition of extracellular regulated kinases 1/2 and protein kinase B was found to ensure the proapoptotic effect. Additionally, the EGFR, an upstream regulator of both kinases, was inhibited indicating that apoptosis is induced by blocking survival- and proliferation-associated signal cascades at the receptor level. In summary, these findings suggest a new therapeutic concept for the treatment of hyperproliferative diseases by combining topical curcumin with UVA or visible light. In particular, the latter avoids the use of carcinogenic irradiation that is part of regular phototherapy.

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INTRODUCTION

Curcuma longa (Zingiberaceae family) is a pharmacologically active plant and spice widely cultivated in tropical regions of Asia and Central America. The yellow spice, turmeric, and extracts for pharmacological use, are usually derived from the ground rhizome. Turmeric is traditionally used as a remedy in native Asian medicine. The main ingredient and active component of turmeric is curcumin (dihydroferuloyl-methane), which contains two phenolic rings, a common structure of many flavonoids, which imparts potent antioxidant activities. Curcumin has been shown to exhibit anti-inflammatory (Ruby *et al.*, 1995; Joe *et al.*, 1997), antitumor, and antioxidative (Ruby *et al.*, 1995; Xu *et al.*, 1997) properties. However, the mechanisms underlying these diverse effects are not yet fully understood.

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Abbreviations: ERK, extracellular regulated kinase; PKB, protein kinase B; PBS, phosphate-buffered saline; TNF, tumor necrosis factor

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Curcumin causes cell proliferation arrest and induces apoptosis in several types of human and animal cells. The initiated cell death pathway seems to depend strongly on the cell type used. Curcumin activation of caspase-8 in gastric and colon cancer cells (Moragoda *et al.*, 2001) and in human melanoma cells (Bush *et al.*, 2001) indicates apoptosis induction via the extrinsic pathway. Additionally, Bush *et al.* (2001) have demonstrated a Fas-receptor activation following curcumin treatment. However, other studies indicate that curcumin treatment induces intrinsic apoptosis. Particularly in breast epithelial cells, curcumin downregulates the antiapoptotic Bcl-2, a protein known to prevent cytochrome *c* release from mitochondria, which is considered to be a key step for the onset of intrinsic apoptosis. *Vice versa*, Bax the proapoptotic counterpart of Bcl-2, becomes upregulated (Kim *et al.*, 2001; Choudhuri *et al.*, 2002). Furthermore, curcumin-induced release of cytochrome *c* was shown in HL-60 (Bielak-Mijewska *et al.*, 2004) and K-562 cells (Chakraborty *et al.*, 2006). It is well known that cellular signaling cascades involved in apoptosis are closely linked to growth control and cell survival. It is demonstrated that curcumin modulates cellular key events in this context. In particular, protein kinase B (PKB/Akt), a pivotal cell survival factor, is inhibited by curcumin in human prostate cancer cells (Chaudhary and Hruska, 2003) and in human HBL100 and MDA-MB-468 breast cell lines (Squires *et al.*, 2003). Likewise, an inhibitory effect on growth-associated mitogen-activated protein kinases (MAPK), such as extracellular

regulated kinase (ERK)1/2 and the c-jun N-terminal kinase (SAPK/JNK), was shown in the aforementioned breast cell lines (Squires *et al.*, 2003) as well as in pancreatic stellate cells (Masamune *et al.*, 2006). In contrast to this, HCT116 human colon cancer cells respond to curcumin by SAPK/JNK activation showing no effect on the activation of ERK1/2 in these cells (Collett and Campbell, 2004). The regulation of cell survival is also documented by the downregulation of the NF-κB (Singh and Aggarwal, 1995; Jobin *et al.*, 1999). Taken together, curcumin induces antiproliferative as well as proapoptotic effects in different cell species, whereas the mode of action seems to depend on the cell type.

This study is driven by the observation that transdermal and oral application of curcumin is hampered by a low absorption rate. The oral administration in rodents (Ravindranath and Chandrasekhara, 1980; Ireson *et al.*, 2001) as well as in humans (Shoba *et al.*, 1998) resulted in negligible amounts of curcumin detection in plasma. Therefore, strategies that allow an increased bioeffectivity are favored. Inspired by studies utilizing bacteria and a rat basophilic leukemia-cell model showing phototoxic effects of curcumin (Dahl *et al.*, 1989, 1994), we tested whether the combination of light and curcumin induces growth arrest and apoptosis in skin cells.

RESULTS

Cellular uptake and intracellular distribution of curcumin

Cellular uptake of curcumin was observed in HaCaT keratinocytes incubated with 5 μg/ml curcumin after different time intervals ranging from 0 minute to 6 hours in cell culture medium without phenol red. Methanolic extracts were photometrically measured for curcumin as described. It was found that curcumin rapidly penetrated the cells (Figure 1a). After only 15 minutes, a significant amount was found within the cells. After 45 minutes, a maximum of intracellular curcumin was reached followed by a slow but steady decrease of optical density. To trace the subcellular distribution, curcumin-treated cells were examined by confocal laser scanning microscopy. Figure 1b shows curcumin-derived fluorescence accumulated in the cytoplasmic granula located close to the nucleus after 40 minutes. This cellular distribution was not changed within the observation period (4 hours) nor after treatment with UVA. Interestingly, the nucleus itself showed no fluorescence, indicating that curcumin and its fluorescing derivates do not enter the nucleus.

Curcumin combined with UVA- or visible light inhibited cell proliferation

HaCaT keratinocytes were preincubated with curcumin (0.1–1 μg/ml) for 1 hour and then irradiated with UVB (100 mJ/cm^2), UVA (1 J/cm^2), or visible light (5,500 lx, 5 minutes). After 24 hours, the incorporation rate of BrdU was measured as described. Using an energy dose up to 100 mJ/cm^2, UVB had no effect on DNA synthesis. The measured data did not change between control cells grown with curcumin alone or cells irradiated with UVB alone (data not shown). Irradiation of curcumin-treated cells with 1 J/cm^2 UVA or visible light gave rise to a distinct inhibition of cell proliferation in both HaCaT cells (Figure 2a) and normal keratinocytes (Figure 2b). Irradiation with 5,500 lx visible light and curcumin was carried out for 5–20 minutes (data not shown). For further experiments, we used an irradiation time of 5 minutes, which gave comparable results as 1 J/cm^2 UVA. The rate of inhibition depended on the concentration of

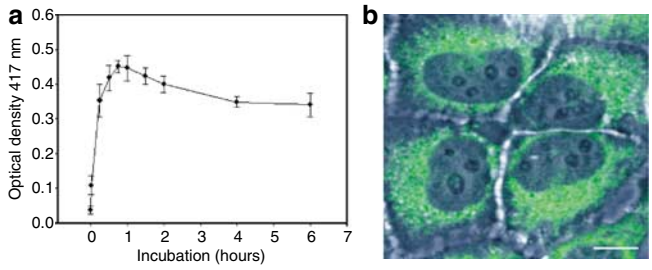

Figure 1. Cellular uptake and intracellular distribution of curcumin. HaCaT cells were incubated with 5 μg/ml curcumin. (**a**) The uptake was determined by measuring the optical density of methanolic cell extracts after 0–6 hours. Each value represents the mean of three independent experiments. (**b**) The fluorescent distribution is shown by confocal laser-scanning microscopy after 40 minutes. Bar = 5 μm. Experiments were repeated with similar results.

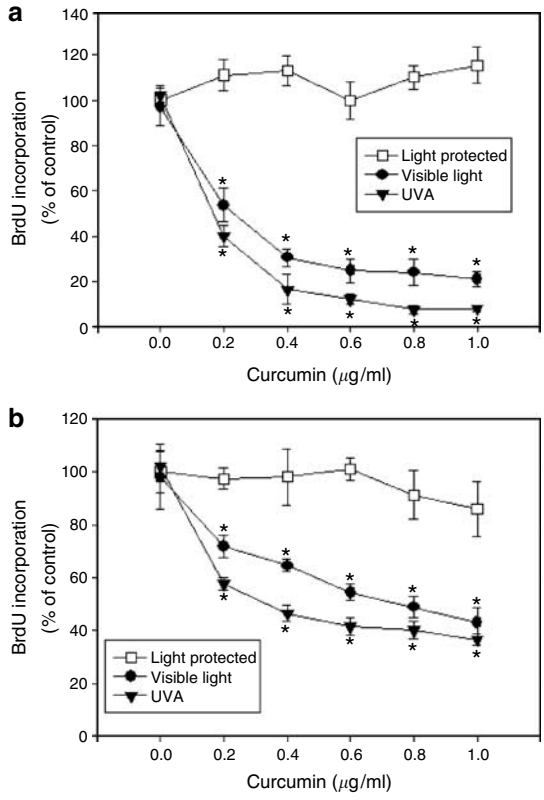

Figure 2. Curcumin combined with UVA or visible light inhibits cell proliferation. (**a**) HaCaT cells and (**b**) primary kerationcytes were preincubated with curcumin and irradiated in the presence of curcumin as described in Materials and Methods with 1 J/cm^2 UVA (▼), or 5,500 lx visible light (5 minutes) (●), or kept light protected (□). BrdU incorporation was determined after 24 hours. The values of the control were set to 100%. Each bar represents the mean of five independent experiments (*$P<0.005$ *vs* untreated control). The experiment was repeated with similar results.

curcumin and was at first visible at 0.2 μg/ml. In light-protected but curcumin-treated cultures, no significant proliferation-inhibiting effect was detectable in the concentration range tested. To show whether the light-depending effect is cell specific, we repeated experiments using different cell species, including primary fibroblasts, melanocytes, melanoma cells (MMLI, G-361), and A-431 cells. An inhibition of proliferation by a combination of curcumin and light was also observed in all the tested cells. Light-protected culture did not produce changes in DNA synthesis (data not shown).

Curcumin combined with UVA did not induce ROS

Photosensitizing compounds such as psoralen are known to offer phototoxicity by generation of reactive oxygen species (ROS) (Parrish *et al.*, 1974). To study whether curcumin utilizes a similar mode of action, we investigated the generation of ROS superoxide and hydrogen peroxide after curcumin (0.2–1 μg/ml) and 1 J/cm^2 UVA treatment. No significant induction of ROS was detected by treatment with curcumin alone and a combination of curcumin and UVA (data not shown). To support these findings, we treated HaCaT cells with curcumin (0.2–1 μg/ml) and high doses of UVA (20 J/cm^2) (Figure 3a). Our results showed that curcumin reduced UVA-induced ROS in a concentration-dependent manner. In contrast to curcumin, psoralen (plus 20 J/cm^2 UVA) caused a concentration-dependent increase of ROS (Figure 3b). We conclude that curcumin/light utilizes a mechanism different from psoralen, the prototypical photosensitizer used in dermatology. In contrast, curcumin offered radical scavenger potency. Different energy doses of UVA ranging from 0.5 to 20 J/cm^2 were tested to evaluate the effect of UVA alone on ROS generation (Figure 3c). These results clearly demonstrated that 1 J/cm^2 UVA, which was used in our other experiments, has no effect on ROS generation.

Curcumin combined with light did not induce toxic membrane damages

To investigate whether the observed proliferation-inhibiting effect of curcumin in the presence of light is due to toxic membrane damages, we incubated HaCaT keratinocytes with different concentrations of curcumin (0.05–1 μg/ml) and irradiated the cells with 1 J/cm^2 UVA. After 24 hours, the release of lactate dehydrogenase in cell culture supernatants was measured. Cell membrane integrity was not changed by curcumin alone and also not by a combination of UVA and curcumin up to 1 μg/ml (Figure 4).

Curcumin combined with light induced apoptotic bodies

In addition to the inhibition of DNA synthesis, a combination of light (UVA) and curcumin was tested to see if it triggers apoptosis. Thus, cells were stained with bisbenzimide to observe the formation of apoptotic bodies (Wyllie *et al.*, 1984) as described. We found an increase of apoptotic nuclei in HaCaT cells 24 hours after treatment with UVA and 1 μg/ml curcumin (Figure 5a and b). More than 40% of treated cells (1 μg/ml curcumin, 1 J/cm^2 UVA) showed apoptotic nuclei compared with light-protected controls, which showed only 0.5% of cells with apoptotic bodies (Figure 5c).

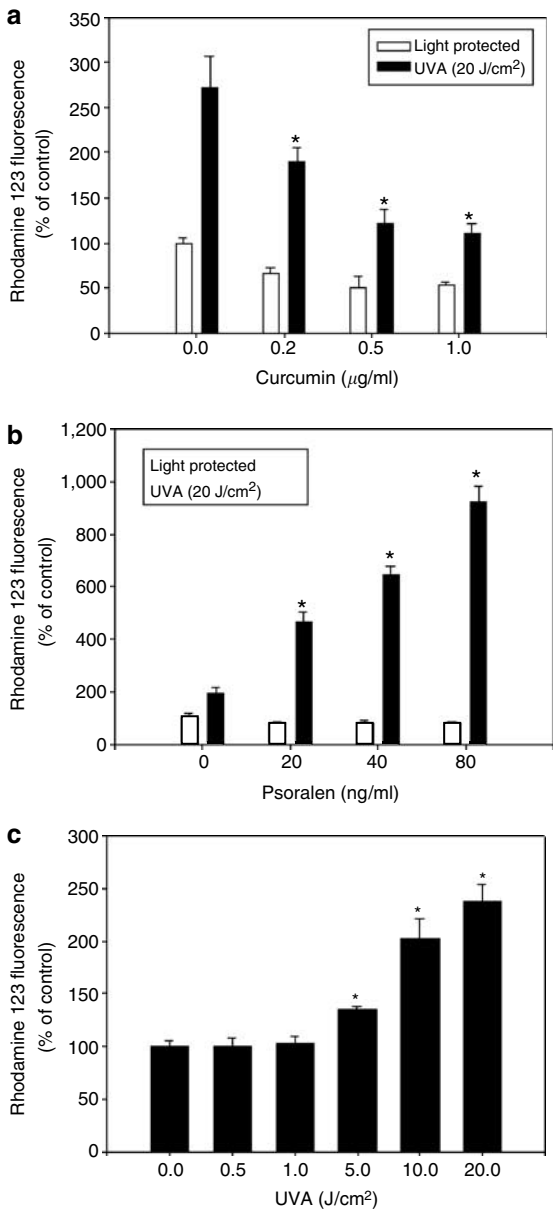

Figure 3. Curcumin combined with UVA does not induce ROS. HaCaT cells were preincubated with dihydrorhodamine and (**a**) curcumin or (**b**) psoralen as described in Materials and Methods and then irradiated with 20 J/cm^2 UVA (black bars) or kept light protected (white bars). Data measured after 90 minutes are shown. (**c**) HaCaT cells were irradiated with 0.5–20 J/cm^2 and rhodamine 123 fluorescence was determined as described above. The values of the control were set to 100%. Each bar represents the mean of eight independent experiments (*$P<0.005$ *vs* (**a**), (**b**) irradiated or **c** untreated control). Experiments were repeated with similar results.

Curcumin combined with light induced cytochrome *c* release

The release of cytochrome *c* from mitochondria is an essential step in the intrinsic apoptotic pathway (Green and Reed, 1998). To determine whether a combination of light (UVA or visible light) and curcumin induces the release of

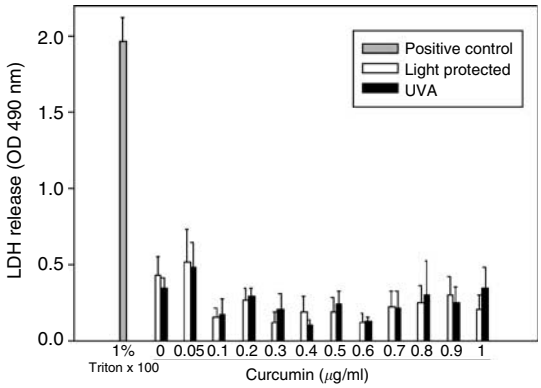

Figure 4. Curcumin combined with UVA does not induce toxic membrane damage. HaCaT cells were preincubated with curcumin and irradiated in the presence of curcumin as described in Materials and Methods with 1 J/cm^2 UVA (black bars) or kept light protected (white bars). After 24 hours, lactate dehydrogenase (LDH) of the cell-free supernatants was measured. Supernatants of cells treated with 1% Triton X100 (gray bars) served as a positive control. Each bar represents the mean of three independent experiments. The experiment was repeated with similar results.

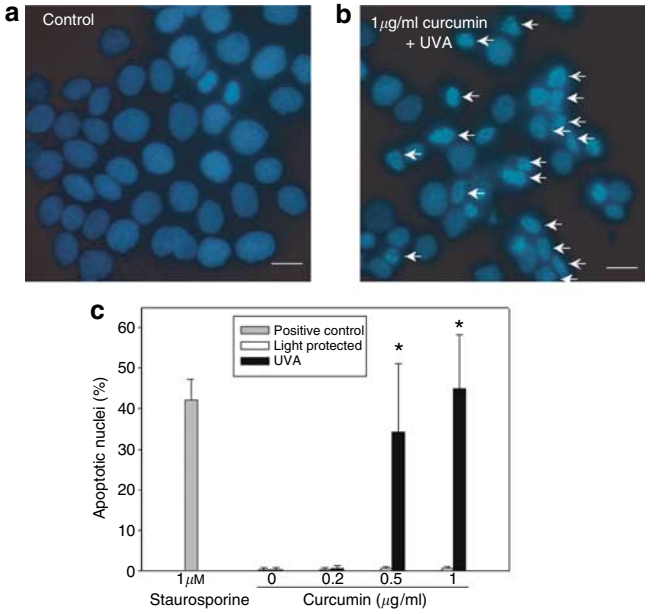

Figure 5. Curcumin combined with UVA induces apoptotic bodies. HaCaT cells were preincubated with curcumin, then (**a**) kept light protected or (**b**) were irradiated with 1 J/cm^2 UVA. After 24 hours, bisbenzimide staining was performed. Apoptotic bodies are marked with arrows. (**c**) The evaluation was carried out by counting approximately 200 cells of each probe of irradiated (black bars), or light protected (white bars) as well as 1 μM staurosporine-treated cells (positive control; gray bar) (*$P<0.05$ *vs* untreated control). Bar = 10 μm. The experiment was repeated with similar results.

cytochrome *c* from mitochondria, cells were pretreated for 1 hour with different concentrations of curcumin, and then irradiated with 1 J/cm^2 UVA or visible light as described. After 24 hours, cell extracts were prepared and cytoplasmic cytochrome *c* was quantified as described. Figure 6 shows that curcumin in combination with UVA or visible light

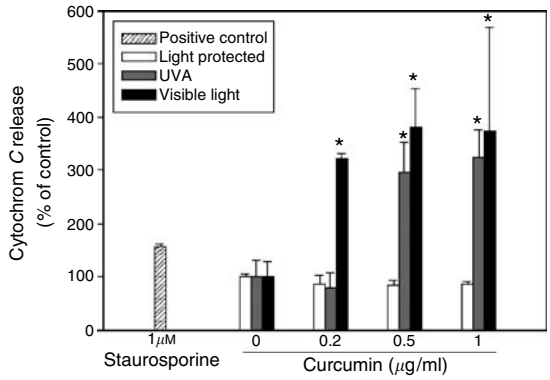

Figure 6. Curcumin combined with UVA or visible light enhances the release of cytochrome *c*. HaCaT cells were preincubated with curcumin and irradiated in the presence of curcumin as described in Materials and Methods with 1 J/cm^2 UVA (gray bars), or 5,500 lx visible light (5 minutes) (black bars) or kept light protected (white bars). The positive control was incubated with 1 μM Staurosporine (striped bar). After 24 hours, cytoplasmic extracts were obtained and cytochrome *c* determination was performed. The values of the control were set to 100%. Each bar represents the mean of three independent experiments (*$P<0.05$ *vs* untreated control). The experiment was repeated with similar results.

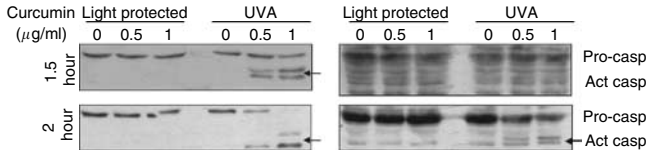

Figure 7. Curcumin combined with UVA activates caspases. HaCaT cells were preincubated with curcumin and irradiated in the presence of curcumin as described in Materials and Methods with 1 J/cm^2 UVA (lanes 4–6) or kept light protected (lanes 1–3). After 1.5 and 2 hours, proteins were obtained and Western blotting was performed. The blots show the inactive procaspases and the cleaved forms (black arrows) of (left panel) caspase-9 and (right panel) caspase-8. The experiment was repeated with similar results.

induced a concentration-dependent release of cytochrome *c*. Light-protected cells showed no increase in cytoplasmic cytochrome *c* in response to curcumin up to 1 μg/ml. At 0.2 μg/ml, curcumin induced a massive liberalization of cytochrome *c* in combination with visible light, whereas UVA offered no effect at this concentration. At higher concentrations (0.5 and 1 μg/ml) visible light and UVA induced similar effects on the release of cytochrome *c*. Interestingly, curcumin/light treatment offered stronger effects on cytochrome *c* release than on staurosporine, a well-known inductor of apoptosis, serving as positive control in this assay.

Curcumin combined with light activated caspases

To confirm the induction of the mitochondrial-mediated apoptosis, the activation of the intrinsic initiator caspase-9 was examined. HaCaT keratinocytes were treated with different concentrations of curcumin for 1 hour, and then irradiated with 1 J/cm^2 UVA as described. After 1.5 and 2 hours, cell extracts were analyzed by Western blotting and probed against caspase-9 (Figure 7, left panel). Curcumin together with UVA induced an activation of caspase-9 documented by the cleavage of the inactive full-length

caspase-9 and by the appearance of cleaved fragments after only 1.5 hours. To test whether the extrinsic apoptosis pathway is also involved in curcumin-induced apoptosis, caspase-8 was examined (Figure 7, right panel). We could show that a combination of UVA and curcumin also activated caspase-8, indicated by cleavage of the inactive procaspase. Interestingly, we demonstrated a time-dependent dichotomy between intrinsic and extrinsic apoptosis indicated by no appearance of caspase-8 fragments until 2 hours, which is at least 30 minutes later than in the case of caspase-9. Light-protected controls did not show any curcumin-induced caspase activation in the concentration range tested.

Curcumin combined with light-inhibited NF-κB

The NF-κB transcription factor is present in the cytoplasm in an inactive state, bound to the inhibitory IκB protein (Baeuerle *et al.*, 1988). Activation occurs via phosphorylation of IκB, resulting in the release of active NF-κB (Brockman *et al.*, 1995; Traenckner *et al.*, 1995). It is well known that curcumin, at high concentrations from 50 to 150 μg/ml, inhibits the prosurvival transcription factor NF-κB in various cell types (Singh and Aggarwal, 1995; Jobin *et al.*, 1999). To investigate whether low curcumin concentrations, combined with light, have an effect on NF-κB in HaCaT keratinocytes, a transactivation assay was performed. Both UVA and visible light strongly inhibited NF-κB transactivation in combination with curcumin (0.2–1 μg/ml) in a concentration-dependent manner (Figure 8a). In light-protected controls, only a slight increase of luciferase activity was detected. The positive control, tumor necrosis factor (TNF)-α, enhanced the NF-κB activity up to 160% compared with untreated controls. To support these findings, NF-κB electrophoretic mobility shift assay was carried out (Figure 8b). To detect inhibitory effects of curcumin/UVA, cells were additionally stimulated with 10 ng/ml TNF-α. Results showed a concentration-dependent decrease of NF-κB binding activity 30 minutes after curcumin and UVA treatment. Concentrations of 0.5 and 1 μg/ml curcumin in combination with UVA resulted in complete inhibition. Same nuclear extracts were subjected to Western blot analysis against NF-κB p65 and cytosolic extracts against IκB-α (Figure 8c). Curcumin/UVA treatment showed a clear increase of cytosolic IκB-α in a concentration-dependent manner, whereas TNF-α-induced translocation of NF-κB to the nucleus was suppressed.

Curcumin combined with light inhibited PKB/Akt, ERK1/2, and EGF receptor

As the intrinsic apoptosis pathway is particularly linked to PI3/PKB as well as MAPK signaling pathways, we investigated the effect of UVA in combination with curcumin on the growth/survival-associated kinases PKB/Akt, ERK1/2, and the upstream receptor of both, the EGF-R. No activation of the basal levels of PKB/Akt, ERK1/2, and EGF-R was detected after curcumin and light (UVA) treatment (data not shown). In the following, it was tested if this treatment has an inhibitory effect. Therefore, cells were stimulated with 100 ng/ml TGF-α

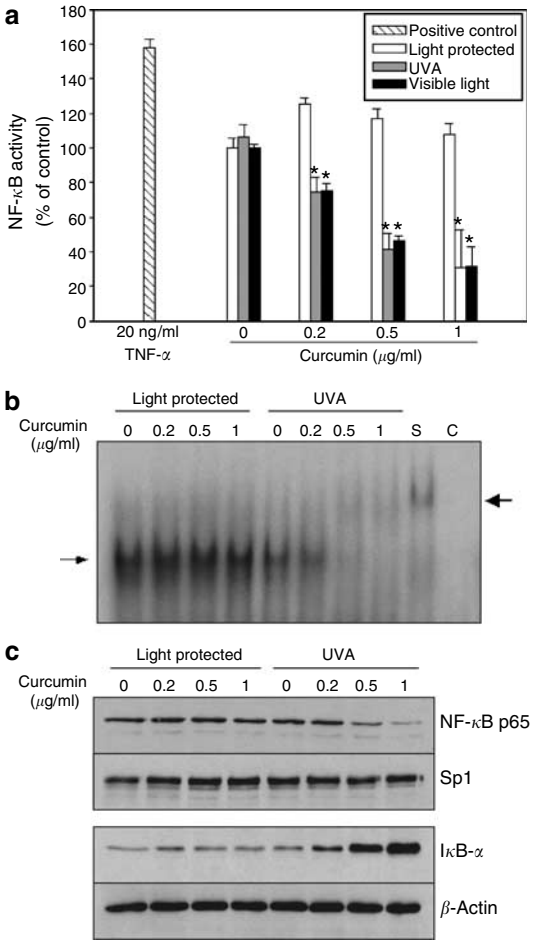

Figure 8. Curcumin combined with UVA or visible light inhibits NF-κB. (**a**) HaCaT cells were cotransfected with NF-κB-binding domain promoter as well as with an SV40 promotor plasmid. The next day, cells were preincubated with curcumin and irradiated in the presence of curcumin as described in Materials and Methods with 1 J/cm^2 UVA (gray bars), or 5,500 lx visible light (5 min) (black bars) or kept light protected (white bars). The positive control was incubated with 20 ng/ml TNF-α (striped bar). After 24 hours, cell lysates were obtained and NF-κB transactivation assay was performed. The values of the control were set to 100%. Each bar represents the mean of four independent experiments (*$P<0.05$ *vs* untreated control). The experiment was repeated with similar results. (**b** and **c**) HaCaT cells were treated with curcumin and UVA as described above. After the addition of 10 ng/ml TNF-α for 30 minutes, nuclear and cytosolic proteins were extracted. (**b**) Electrophoretic mobility shift assay was carried out with nuclear extracts of curcumin-treated (lanes 1–4) and curcumin/UVA-treated (lanes 5–8) HaCaT cells and ^{32}P-labeled NF-κB consensus sequence. Competition (C) and supershift using a specific NFκB antibody (S) were performed as described in Materials and Methods. (**c**) Amounts of nuclear NFκB (p65) were detected by Western blot analysis. As loading control served Sp1. Cytosolic extracts of the same samples were used for detection of IκB-α. As loading control served β-actin. Experiments were repeated with similar results.

to induce a maximum phosphorylation of the kinases tested (Figure 9). Western blot analysis revealed a dose-dependent inhibition of phosphorylation of all three investigated parameters, PKB/Akt (Figure 9a), ERK1/2 (Figure 9b), and EGF-R (Figure 9c) after curcumin/UVA treatment. In light-protected cultures, this effect was not observed.

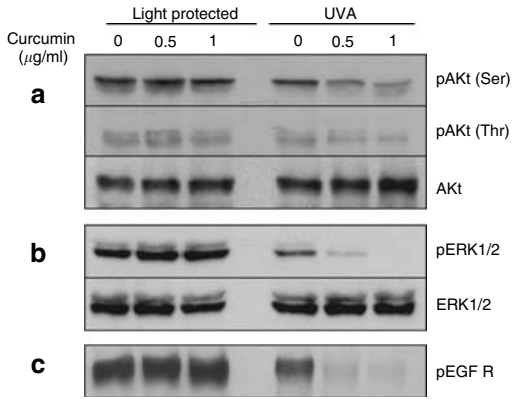

Figure 9. Curcumin combined with UVA inhibits PKB/Akt, ERK1/2, and EGF-R. HaCaT cells were preincubated with curcumin and irradiated in the presence of curcumin as described in Materials and Methods with 1 J/cm^2 UVA (lanes 4–6) or kept light protected (lanes 1–3). Then 100 ng/ml TGF-α was added to the cells. After 15 minutes, proteins were obtained and Western blotting was performed with antibodies directed toward (**a**) phosho-PKB/Akt (Ser), phospho-PKB/Akt (Thr), PKB/Akt, (**b**) phospho-ERK1/2, ERK1/2, and (**c**) phospho-EGF-R. Detection of total PKB/Akt and ERK1/2 served as loading control. The experiment was repeated with similar results.

DISCUSSION

Growth inhibition and apoptosis by curcumin are well documented for many different cells suggesting curcumin as a potent drug against hyperproliferative diseases. However, medical application of curcumin is hampered by minor gastrointestinal resorption. After oral administration of 400 mg curcumin to rats, less than 5 μg/ml was detected in blood and 20 μg/tissue in the liver and kidney (Ravindranath and Chandrasekhara, 1980). Similarly, tests with human volunteers showed almost no compound traces within the serum after intake of 2 g curcumin (Shoba *et al.*, 1998). *In vitro* studies demonstrated that concentrations of 10–150 μM (3.7–55 μg/ml) are necessary to show pharmacological effects (Jobin *et al.*, 1999; Choudhuri *et al.*, 2002). Therefore, options to enhance the bioavailability of curcumin are desirable.

Inspired by the observation from Dahl *et al.* (1989, 1994) showing that curcumin offers phototoxic effects, we hypothesized that curcumin-induced growth arrest and apoptosis is amplified by light. In this study, using different skin cells, we showed that curcumin at 0.2 and 1 μg/ml – which is more than one magnitude lower than the lowest concentration providing pharmacological effects – causes proliferation inhibition without toxic membrane damages and induction of ROS when combined with UVA or visible light.

Furthermore, such treatment induced apoptosis in HaCaT cells indicated by the formation of apoptotic bodies, release of cytochrome *c*, and activation of caspase-9 and -8. Release of cytochrome *c* from mitochondria marks the onset of intrinsic apoptosis and is triggered by proteins of the Bcl-2 family (Reed *et al.*, 1998). Subsequent activation of caspase-9 followed by delayed caspase-8 activation confirmed the intrinsic apoptosis signaling cascade as shown by Slee *et al.* (1999). Other studies using curcumin alone at pharmacological effective concentrations also demonstrated the onset of intrinsic apoptosis indicated by regulation of Bcl-2 proteins (Kim *et al.*, 2001; Chaudhary and Hruska, 2003) and the release of cytochrome *c* (Bielak-Mijewska *et al.*, 2004; Chakraborty *et al.*, 2006) in HL-60 and K-562 cells. Of note, in human melanoma cells curcumin seemed to favor extrinsic apoptosis via activation of the Fas receptor (Bush *et al.*, 2001). Similarly, in human renal cancer cells, another transmembrane receptor, the death receptor 5, was modulated by curcumin (Jung *et al.*, 2005). These findings indicate that the route of apoptosis induced by curcumin is specific to cells and tissues.

Singh and Aggarwal (1995) first demonstrated curcumin as a potent inhibitor of the survival factor NF-κB in human myeloid cells (ML-1a), which was later proven in various other studies in other cell species (Jobin *et al.*, 1999; Bharti *et al.*, 2003; Aggarwal *et al.*, 2004). This study does confirm NF-κB inhibition, but, interestingly, at low concentrations (0.2–1 μg/ml) when combined with light. It seems likely that the inhibition of NF-κB facilitates apoptosis as shown by other studies (Barkett and Gilmore, 1999).

Furthermore, we demonstrated that curcumin at low concentration inhibits the proliferation-associated MAPKs ERK1/2 in combination with light. Using higher concentrations in the absence of light had the same effect in breast cells (Squires *et al.*, 2003). Similarly, PKB/Akt, a key molecule of cell survival, was inhibited by curcumin at high concentrations (Chaudhary and Hruska, 2003; Squires *et al.*, 2003). Similarly, our results showed suppression of PKB/Akt at low concentrations of curcumin when combined with UVA in HaCaT cells. PKB/Akt is activated by protein tyrosine kinase-receptors such as the EGF-R. In addition to the study by Squires *et al.* (2003) and Kim *et al.* (2006), which showed inhibition of the EGF-R by curcumin, we demonstrated light-induced sensitization of curcumin effects. These results suggest that curcumin may induce apoptosis by inhibiting the EGF-R and the downstream targets ERK1/2 and PKB/Akt. The latter gives rise to activation of Bad (Datta *et al.*, 1997; del Peso *et al.*, 1997), which thereby translocates to mitochondria causing the release of cytochrome *c*. While this paper was in preparation, Koon *et al.* (2006) showed inhibition of mitochondrial activity by curcumin in combination with light.

In summary, our study shows that a combination of light and curcumin amplifies the proapoptotic effects of curcumin. This observation suggests a novel approach in photodynamic therapy.

MATERIALS AND METHODS

Cell culture

The spontaneously immortalized human keratinocyte cell line HaCaT (Boukamp *et al.*, 1988) was kindly provided by Dr. Norbert Fusenig (German Cancer Research Institute, Heidelberg, Germany). HaCaT cells were cultured in carbonate-buffered Hank's medium supplemented with 5% fetal calf serum (PAA, Coelbe, Germany) and 1% penicillin–streptomycin (Biochrom KG, Berlin, Germany) at 37°C in a 5% CO_2 atmosphere. Primary human keratinocytes were propagated from skin samples provided from the Department of

Plastic and Reconstructive Surgery (Diakonie Hospital, Bad Kreuznach, Germany), with patient consent. Primary keratinocytes were isolated as described by Stein *et al.* (1997). Briefly, the skin specimens were washed with phosphate-buffered saline (PBS), minced, and treated with 0.25% trypsin solution overnight at 4°C. Subsequently, epidermis was separated from dermis and the epidermis was trypsinized for another 15 minutes at room temperature. The suspension was filtered through sterile gauze and plated in serum-free keratinocyte medium KGM-2 (Cell Systems, St Katharinen, Germany) with 1% penicillin–streptomycin solution at 37°C and 5% CO_2. All studies were conducted according to the Declaration of Helsinki Principles and in agreement with the Local Ethic Commission.

Uptake kinetic and intracellular distribution of curcumin

The uptake kinetic of curcumin was determined, according to the method of Dahl *et al.* (1994). HaCaT keratinocytes were cultivated in six-well multidishes (1.5×10^6 cells/9.6 cm^2) with 5 μg/ml curcumin (Sigma, Deisenhofen, Germany) in Hank's medium without phenol red for different time intervals (0–6 hours) at 37°C. Then, medium was decanted and dishes were rinsed with PBS. Subsequently, 500 μl methanol were added to each dish and swirled over the cell layer. Methanol extracts were collected and their optical density was measured at 415 nm in a scanning multiwell spectrophotometer (ELISA reader MR 5000, Dynatech, Guernsey, UK). Intracellular distribution of curcumin-derived fluorescence was investigated in living cells. HaCaT keratinocytes were incubated with 5 μg/ml curcumin (Sigma) in phenol red-free Hank's medium. Cells were observed using a laser-scanning microscope (420 nm; Zeiss, Oberkochen, Germany) and Imaris software (Bitplane, Zürich, Switzerland).

Curcumin treatment and irradiation

Thirty milligrams of curcumin (Sigma) was dissolved in 1 ml DMSO. The stock solution was diluted in Hank's medium or PBS to concentrations ranging from 0.1 to 1 μg/ml. Cells were incubated for 1 hour with medium containing curcumin at 37°C in a 5% CO_2 atmosphere. The cells were irradiated with 1 J/cm^2 UVA or 100 mJ/cm^2 UVB, respectively (UV therapy system UV 3003K; Waldmann, Villingen-Schwenningen, Germany). Emitted light ranges were 315–400 nm with a maximum at 365 nm in the case of UVA and 285–350 nm with a maximum at 310–315 nm in the case of UVB. They did not emit any biologically significant amounts of UVC, which was controlled using a 254-nm detector (UV-meter; Kurt Migge Laborbedarf, Heidelberg, Germany). To avoid the formation of phototoxic products within the culture medium, PBS containing curcumin was used during irradiation. Cells were also irradiated for 5 minutes with 5,500 lx visible light (10×40 W lamps, distance 45 cm, emission spectrum: 400–550 nm; Philips GmbH, Hamburg, Germany). Non-irradiated controls were kept in the dark.

BrdU incorporation

To determine the replication rate, cells were cultured in microwell plates at a density of 2×10^4 cells per 0.33 cm^2. After 24 hours, cultures were treated with curcumin and light as aforementioned. Subsequently, BrdU was added to the cells. The incorporation rate of BrdU was determined after 24 hours using a commercial ELISA test kit (Roche, Mannheim, Germany). Briefly, cells were fixed and immune complexes were formed using peroxidase-coupled BrdU antibodies. A colorimetric reaction with tetramethylbenzidine as a substrate gave rise to a reaction product measured at 450 nm using an ELISA reader (MR 5000, Dynatech).

Monitoring ROS generation

Free radical generation was monitored by following the oxidation of dihydrorhodamine 123, an oxidation-sensitive indicator, which can be converted to the fluorescent derivative rhodamine 123, namely by superoxide (Henderson and Chappell, 1993) and hydrogen peroxide (Royall and Ischiropoulos, 1993). Ten milligrams of dihydrorhodamine 123 (Sigma) were dissolved in 1.5 ml ethanol, and 28.9 ml PBS was added to the solution. HaCaT cells cultured in microwell plates (2×10^4 cells/0.33 cm^2) were given 10 μM dihydrorhodamine 123 together with curcumin or psoralen. After 1 hour, cells were washed with PBS and irradiated with UVA. Consecutively, rhodamine 123 fluorescence was measured every 10 minutes (excitation 485 nm, emission at 530 nm) using a CytoFluor (series 4000, PerSeptive Biosystems, Framingham, MA). Maximum fluorescence was reached after 90 minutes.

Cytotoxicity

Cell death and cell lysis were quantified using the cytotoxicity detection kit (Roche), which is based on release of lactate dehydrogenase from damaged cells. Briefly, HaCaT cells were cultivated in microwell plates (2×10^4 cells/0.33 cm^2) and treated with curcumin and light as aforementioned. Next day cell-free supernatants were incubated with NAD^+, which becomes reduced by lactate dehydrogenase to $NADH/H^+$. Consecutively, $NADH/H^+$ reduces the yellow tetrazolium salt to a red-colored formazan salt. The amount of red color is proportional to the number of lysed cells. For quantification, the absorbance of the reaction product was measured at 490 nm using an ELISA reader (MR 5000, Dynatech).

Nuclei staining

Nuclear morphology was examined using the fluorescent DNA stain bisbenzimide H 33342, (Riedel de Häen, Seelze, Germany). Then, 1.4×10^4 cells/1.7 cm^2 were plated in four-well LabTeks (Nunc, Wiesbadon, Germany) and treated the next day with curcumin and light as described. After washing with PBS, cells were fixed with methanol/acetone (1:1 vol/vol) for 30 minutes. After another washing step with PBS, cells were stained with bisbenzimide (1 μg/ml) at 37°C for 30 minutes. Consecutively, slides were washed three times with PBS and mounted with Aquatex (Merck, Darmstadt, Germany). Nuclei were examined by light microscopy (Axioscop, Zeiss) and assessed on the basis of changes in nuclear morphology, such as, chromatin condensation and fragmentation (apoptotic bodies). Two to four sections varying from 31 to 137 cells (at least 200 cells of each probe) were observed.

Cytochrome *c* release

Cells were plated in six-well multidishes (Nunc, 1.5×10^6 cells/9.6 cm^2) and treated the next day with curcumin and light as aforementioned. After 24 hours, cytoplasmic extracts were obtained by digitonin permeabilization (Jiang *et al.*, 1999). Briefly, cells were trypsinized (0.125% trypsin/0.1% EDTA) for 10 minutes, and centrifuged at 259 *g* for 10 minutes and the pellet was resuspended in 250 μl PBS. Permeabilization of the membranes was gained by

adding 250 μl digitonin/sucrose (80 μg/ml, Fluka, Buchs, Switzerland) for 30 seconds. Then samples were centrifuged for 1 minute at 17,900 *g* (4°C) and supernatants with equal amounts of protein (protein determination by DC protein assay; BioRad, Munich, Germany) were used in a commercial cytochrome *c* immunoassay kit (R&D Systems, Wiesbaden, Germany). The assay was performed according to the manufacturer's manual. The optical density (450 nm) was measured using an ELISA reader (MR 5000, Dynatech).

NF-κB transactivation assay

Expression plasmid containing firefly luciferase driven by five copies of the NF-κB consensus sequence was kindly provided by Marion Boland (Department of Biochemistry, Dublin, Ireland). Transactivation assays were performed using the dual-luciferase reporter assay (Promega, Mannheim, Germany). Briefly, HaCaT cells were plated in 24-well multidishes (Nunc) and co-transfected after reaching 70% confluence with lipofectamine (LF2000; Invitrogen, Karlsruhe, Germany) with NF-κB firefly luciferase plasmids and renilla luciferase-containing plasmids driven by an SV40 promoter. The latter served to monitor transfection efficiency. The following day, cells were treated with curcumin and light as described and cultivated for a further 24 hours. After cell lysis, activity of both luciferases was detected separately using a luminometer (Lumat LB 9507; Berthold, Bad Wildbad, Germany).

EMSA

Cells were plated in six-well multidishes (1.5×10^6 cells/9.6 cm^2) and treated as described above. After irradiation with 1 J/cm^2, UVA cells were stimulated with 10 ng/ml TNF-α (PreproTech, Umkirch, Germany). Nuclear proteins were extracted as described previously (Dignam *et al.*, 1983). NF-κB binding activity was performed using double-stranded NF-κB consensus oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′; Biospring, Frankfurt/Main, Germany) 5′-labeled with ^{32}P by Hartmann Analytic (Braunschweig, Germany). DNA binding reaction was performed for 20 minutes at room temperature in a volume of 20 μl, containing 8 μg nuclear proteins, 2.5 mg/ml BSA, 10^5 c.p.m. ^{32}P-labeled probe, 0.1 mg/ml poly[dI:dC] (Sigma), and 5 μl of 4 × binding buffer (1 × buffer: 10 mM Tris–HCl, pH 7.8, 100 mM KCl, 5 mM EDTA, 10% (v/v) glycerol, 1 mM dithiothreitol). For the competition control, a 20-fold excess of unlabeled NF-κB consensus sequence and for supershift 1 μg NF-κB p65 antibody (Santa Cruz Biotechnology, Heidelberg, Germany) were added before the addition of the labeled probe. Samples were subjected to electrophoresis on a native 4% PAGE for 2.5 hours at 120 V. The gels were dried and the radioactive bands were visualized by autoradiography.

Western blot analysis

Caspase-8 and -9 activity as well as the phosphorylation status of PKB/Akt, ERK1/2, and EGF-R were detected by Western blot analysis. Cells were plated in six-well multidishes (1.5×10^6 cells/9.6 cm^2) and treated as described above. In the case of positive stimulation, 100 ng/ml TGF-α (PreproTech) was added after irradiation. Cells were lysed with 3 × SDS sample buffer (187.5 mM Tris–HCl (pH 6.8), 6% SDS, 30% glycerol, 150 mM dithiothreitol, 0.3% bromphenol blue), and scraped, centrifuged, and sonicated. Protein extracts were boiled for 5 minutes and separated on a 12% SDS polyacrylamide gel. Nuclear and cytosolic extracts for detection of NF-κB and IκB-α were generated, according to the method described by Dignam *et al.* (1983). For immunoblotting, proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). The membrane was blocked in blocking buffer (Tris-buffered saline (pH 7.6), 0.1% Tween-20, and 5% BSA or non-fat dry milk) for at least 1 hour, followed by overnight incubation with antibodies against activated EGF-R (Transduction Laboratories, Lexington, KY), β-actin (Sigma), Sp1 (Santa Cruz Biotechnology), caspase-8, -9, IκB-α, NF-κB p65, total and phospho-specific PKB/Akt, and ERK1/2 (Cell Signaling Technology, Frankfurt, Germany) in blocking buffer at 4°C. The bound primary antibodies were detected using IgG-horseradish peroxidase-conjugate (Cell Signaling Technology) and visualized with a chemiluminescence detection system (LumiGlo, Cell Signaling Technology).

Statistics

The statistical difference between untreated and treated probes was determined by Mann–Whitney *U*-test. The level of $P<0.05$ was considered to be statistically significant. Data are given as means ± SD.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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