Photosensitizer effect of curcumin on UVB-irradiated HaCaT cells through activation of caspase pathways

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Abstract. Photodynamic therapy (PDT) using photosensitizer can induce diverse cellular responses, including apoptosis. Recently, it has been reported that PDT using methylaminolaevulinate may be effective in basal cell carcinoma. However, it is largely unknown whether PDT using a natural product such as curcumin can induce apoptosis in skin cancer. In this study, to confirm the photodynamic effect of curcumin, we investigated the synergistic effect of the combination of UVB with curcumin on apoptotic cell death in HaCaT cells and molecular mechanisms underlying apoptosis. Our data showed that HaCaT cells were markedly undergoing apoptosis, evidenced by DNA laddering, by combination of UVB with curcumin, compared to UVB or curcumin alone. Furthermore, combination of UVB irradiation with curcumin synergistically induces apoptotic cell death in HaCaT cells through activation of caspase-8, and -3 as well as caspase-9 activation followed by release of cytochrome c. Thus, our data indicate that curcumin may be a promising photosensitizer used in PDT to induce apoptosis in skin cancer cells.

Introduction

Photodynamic therapy (PDT) using photosensitizer can induce diverse cellular responses, including apoptosis (1,2). PDT is becoming an established treatment for selected neoplastic lesions, especially in skin cancer (3-5). PDT using methylaminolaevulinate may be effective in basal cell carcinoma (6,7). However, it is largely unknown whether PDT using natural product such as curcumin can induce apoptosis in skin cancer.

Curcumin is the major constituent of turmeric powder, extracted from the rhizomes of the plant *Curcuma longa*. Curcumin has been described as having antioxidant, anti-inflammatory, and anti-carcinogenic properties (8-11). However, the anti-carcinogenic mechanisms of curcumin action are not fully understood. Curcumin may induce apoptosis of cancer cells through blocking of NF-κB survival pathway, generation of reactive oxygen species (ROS), down-regulation of Bcl-X_L, or activation of caspase-8 pathways (12-14). Recent evidence indicates that curcumin initiates apoptosis through inducing growth arrest and the DNA damage-inducible gene 153 (GADD153), implying curcumin causes DNA damage through topoisomerase II inhibition (15). Expression of GADD153 is up-regulated when cells are exposed to DNA damaging agent including ultraviolet (UV) irradiation. In addition, Dazard *et al* reported that DNA-repair related genes such as GADD45A and GADD45B are markedly up-regulated in UVB-irradiated human keratinocytes (16). Thus, it is important to investigate whether the combination of UVB and curcumin induces synergistically apoptotic cell death in HaCaT keratinocytes through excessive DNA damage, consequently resulting in activation of caspase pathways. In this study, to confirm the photodynamic effect of curcumin, we investigated the synergistic effect of UVB and curcumin on apoptotic cell death in HaCaT cells and molecular mechanisms underlying apoptosis.

Materials and methods

Materials. Antibodies against tubulin, PARP, caspase-3, -8, -9, and cytochrome c were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Curcumin was purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture. Human keratinocyte cell line, HaCaT cells, were maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2 in Eagle's minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin. For experiments, cells ($5x10^4$ cells/ml) were seeded in a culture dish, and maintained in the tissue culture incubator. Cells were preincubated with curcumin for 20 min prior to UVB irradiation at 100 mJ/cm^2.

UVB irradiation. UVB was supplied by a closely spaced array of seven Westinghouse FS-40 sunlamps, which delivered uniform irradiation at a distance of 38 cm. The energy output of UVB (290-320 nm) at 38 cm was measured with a UVB photometer (IL1350 photometer, International Light, Newburyport, MA). Cells were exposed for 0 and 360 sec of UVB, corresponding to doses of 0 and 400 mJ/cm^2. To prevent

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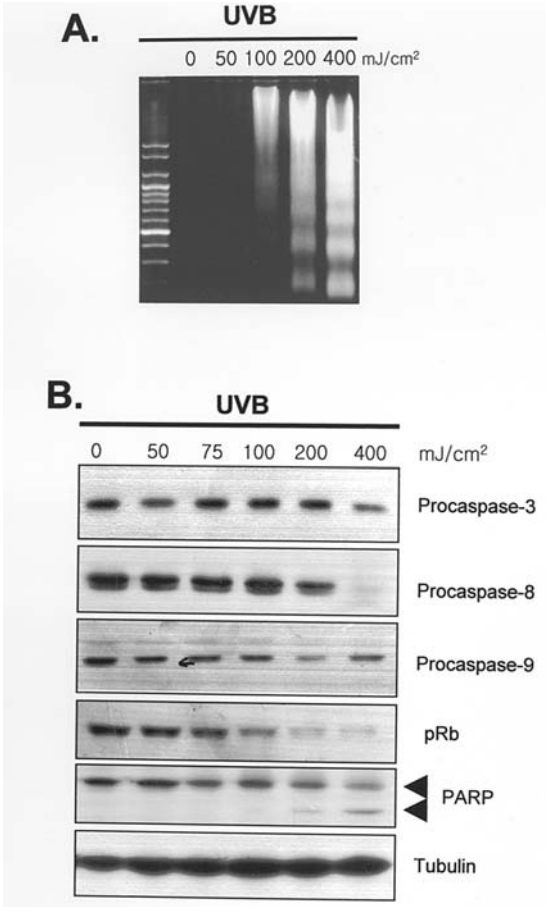

Figure 1. Induction of apoptosis in UVB-irradiated HaCaT cells. Cells were treated by various doses of UVB using seven Westinghouse FS-40 sunlamps. DNA was extracted 24 h after UVB irradiation and analyzed on a 2% agarose gel (A). Proteins were also obtained and subjected to Western blot analysis (B). Experiments were repeated two times and similar results were obtained.

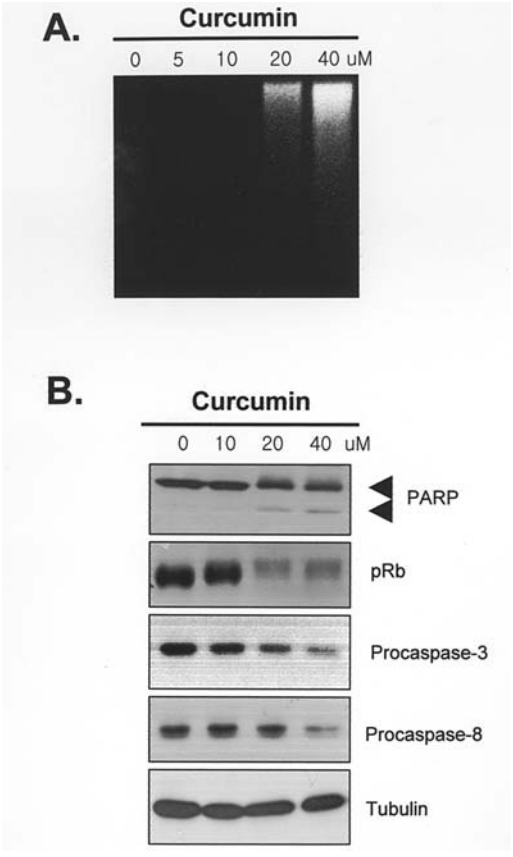

Figure 2. Induction of apoptosis in curcumin-treated HaCaT cells. Cells were treated by various concentrations of curcumin. DNA was extracted 24 h after curcumin treatment and analyzed on a 2% agarose gel (A). Proteins were also obtained and subjected to Western blot analysis (B).

light absorption by tissue-culture medium, the culture medium was removed just prior to irradiation and replaced with a thin layer of phosphate-buffered saline (PBS) to cover the cells. After UVB irradiation, cells were fed with fresh growth medium.

Western blot analysis. Cells were lysed in lysis buffer [10 mM Tris (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton X-100, PMSF (10 μg/ml), aprotinin (10 μg/ml), leupeptin (10 μg/ml), 5 mM phenanthroline, and 28 mM benzamidine-HCl] for 30 min on ice. Lysates were clarified by centrifugation. Lysates were quantitated using the Bradford assay (Life Science Co., CA, USA) with bovine serum albumin as a reference standard. Proteins (35 μg/ml) were resolved by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gels) and transferred to immobilon-P transfer membrane (Millipore Co., MA, USA). After incubation with primary antibodies, proteins were visualized by incubation with horseradish peroxidase-conjugated secondary antibodies, followed by ECL according to the manufacturer's instructions (Amersham Life Science Co., Buckinghamshire, UK).

DNA fragmentation assay. Cells were harvested at the indicated times by centrifugation and lysed in ice for 20 min by the addition of 20 μl lysis buffer consisting of 20 mM EDTA, 100 mM Tris (pH 8.0), and 0.8% (w/v) sodium lauryl sarcosine. RNase A (2 μl, 5 mg/ml) and proteinase K (20 μl, 10 mg/μl) were added and incubated at 37˚C for 1 h and 2 h, respectively. Total lysates were loaded onto 1.5% agarose gel and separated at 50 mV for 2 h. DNA fragments were visualized after staining with ethidium bromide by translumination with UV light.

Results

UVB induces apoptosis in HaCaT cells. To study the effective doses of UVB inducing apoptosis, HaCaT cells were exposed to UVB at doses ranging from 50 to 200 mJ/cm^2, and then cells were harvested 24 h after irradiation for Western blot and DNA laddering assay. As shown in Fig. 1A, distinct apoptotic DNA ladders were observed at >100 mJ/cm^2 of UVB. PARP and pRb cleavages were clearly observed at >200 mJ/cm^2 doses of UVB, concomitantly with caspase-3, -8, and -9 activation (Fig. 1B).

Curcumin enhances UVB-induced apoptosis in HaCaT cells. To study the effective doses of curcumin inducing apoptosis, HaCaT cells were treated with various concentrations of curcumin (5-40 μM), and then cells were harvested 24 h after curcumin treatment for DNA laddering and Western blot assay. As shown in Fig. 2A, distinct apoptotic DNA ladders

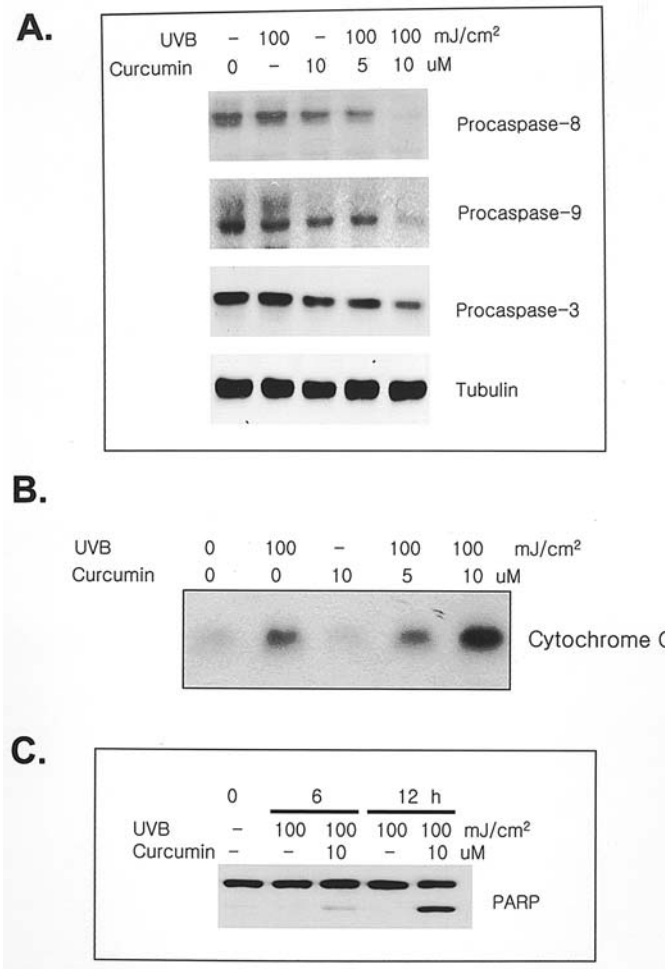

Figure 3. Effect of pretreatment of curcumin on UVB-induced apoptosis. Cells were preincubated with 5 or 10 μM curcumin for 20 min and then exposed to UVB (100 mJ/cm^2). Total proteins and cytosolic fractions were extracted and subjected to immunoblotting using anti-caspases antibodies (A) and cytochrome c antibody (B), respectively. Cells were also preincubated with 10 μM curcumin for 20 min and then exposed to UVB (100 mJ/cm^2). Proteins were extracted at the indicated times and subjected to immunoblotting using anti-PARP antibody. The data are representative of two independent experiments.

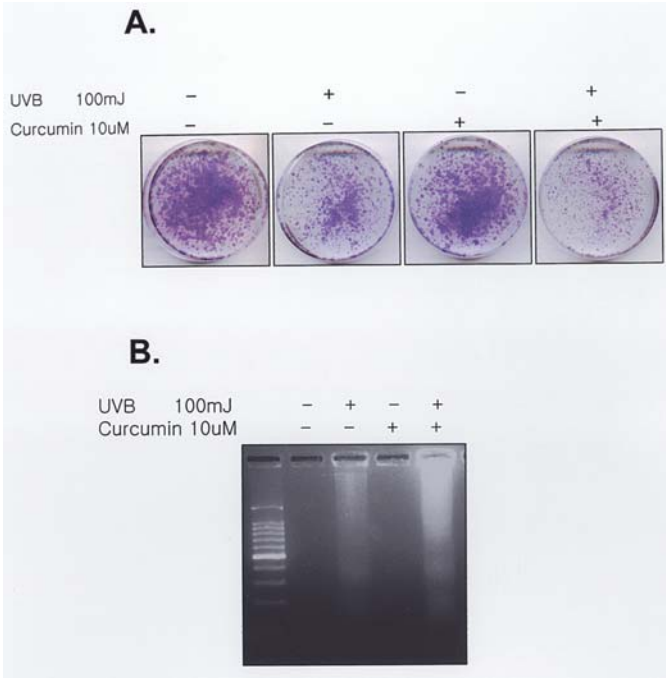

Figure 4. Survival cell colonies and DNA fragmentation assay in combination of curcumin with UVB irradiation. Cells were preincubated with curcumin (10 μM) for 20 min and then exposed to UVB (100 mJ/cm^2). After treatment, cells were cultured for 3 days and then survival colonies were stained using crystal violet staining solution (A). DNA was also extracted 24 h after curcumin and UVB treatment and analyzed on a 2% agarose gel (B).

were observed at >20 μM of curcumin. PARP and pRb cleavages were clearly observed at >20 μM concentration of curcumin, concomitantly with caspase-3 and -8 activation (Fig. 2B).

To study the photodynamic effect of curcumin, HaCaT cells were exposed to sub-apoptotic doses of UVB (100 mJ/cm^2) and cultured in the cultured media including sub-apoptotic concentration of curcumin (5-10 μM), and then cells were harvested 24 h after irradiation for Western blotting. Activation of caspase-3, -8, and -9 were clearly observed in combination of UVB with curcumin, but not in UVB or curcumin alone (Fig. 3A). Furthermore, we observed that the level of cytosolic cytochrome c was higher in UVB with curcumin-treated HaCaT cells than UVB or curcumin-treated cells (Fig. 3B). In agreement with caspase-3 activation and cytochrome c level, PARP cleavages were visualized at 6 h in co-treatment of UVB and curcumin, but not in UVB alone (Fig. 3C).

To determine the synergistic effect of co-treatment of UVB with curcumin on inducing apoptosis, we performed survival colony staining in HaCaT cells 3 days after UVB irradiation with curcumin treatment. Fig. 4A shows that the number of survival colonies was markedly lower in UVB with curcumin-treated HaCaT cells, compared to UVB or curcumin alone. Consistent with the number of survival colonies, distinct DNA fragments were observed in UVB and curcumin co-treated HaCaT cells, compared to UVB or curcumin alone (Fig. 4B).

Discussion

Recently, photodynamic therapy (PDT) has emerged as a promising therapeutic tool for treatment of dermatologic conditions such as *in situ* squamous cell carcinoma, superficial basal cell carcinoma, and malignant melanoma (5,6,17,18). PDT requires the simultaneous presence of a photosensitizer, which accumulates in target cells, and results in induction of apoptosis or necrosis. In this study, our data showed that curcumin may sensitize cells to apoptosis in UVB-irradiated cells through activation of caspase pathways.

Curcumin is a potent chemopreventive agent inhibiting tumor promotion against skin, oral, intestinal and colon carcinogenesis (11,19,20). Our data showed that curcumin induces apoptosis of HaCaT cells, evidenced by DNA fragmentations, caspase-3 and -8 activation, in a concentration-dependent manner. At concentrations >20 μM, there was significant cell death up to ~40 μM concentration. In this study, clear reductions of procaspase-8 and -3 were observed in

combination of UVB with sub-apoptotic concentration (10 μM) of curcumin in HaCaT cells, compared to UVB or curcumin alone, suggesting that apoptosis is effectively induced by co-treatment of UVB with curcumin through caspase-8 and -3 activation. Furthermore, release of cytochrome c and reduction of procaspase-9 were more prominent in combination of UVB with sub-apoptotic concentration (10 μM) of curcumin, compared to UVB or curcumin alone, suggesting that apoptosis is also mediated by mitochondrial-mediated signal pathways. Consistent with our data, Chendil *et al* reported that curcumin confers radiosensitizing effect in prostate cancer cell line PC-3 through activation of cytochrome c and caspase-9 (21). Koon *et al* reported that cytotoxicity of curcumin was enhanced by the irradiation of visible light and blue-filtered light in nasopharyngeal cancer cell lines (22). The possible molecular mechanisms of phototoxicity of curcumin might be that curcumin photogenerates singlet oxygen and reduced forms of molecular oxygen as well as inducing the GADD153 gene (23,24).

Thus, our data suggest that curcumin may be developed as a potential photosensitizer, and a chemotherapeutic agent in clinical application, especially in skin cancer.

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