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Anti-Apoptotic Effects of Curcumin on Photosensitized Human Epidermal Carcinoma A431 Cells

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Abstract Photodynamic treatment (PDT) can elicit a diverse range of cellular responses, including apoptotic cell death. Previously, we showed that PDT stimulates caspase-3 activation and subsequent cleavage and activation of p21-activated kinase 2 (PAK2) in human epidermal carcinoma A431 cells. Curcumin, the yellow pigment of *Curcuma longa*, is known to have anti-oxidant and anti-inflammatory properties. In the present study, using Rose Bengal (RB) as the photosensitizer, we investigated the effect of curcumin on PDT-induced apoptotic events in human epidermal carcinoma A431 cells. We report that curcumin prevented PDT-induced JNK activation, mitochondrial release of cytochrome c, caspase-3 activation, and cleavage of PAK2. Using the cell permeable dye DCF-DA as an indicator of reactive oxygen species (ROS) generation, we found that both curcumin and ROS scavengers (i.e., L-histidine, a-tocopherol, mannitol) abolished PDT-stimulated intracellular oxidative stress. Moreover, all these PDT-induced apoptotic changes in cells could be blocked by singlet oxygen scavengers (i.e., L-histidine, a-tocopherol), but were not affected by the hydroxyl radical scavenger mannitol. In addition, we found that SP600125, a JNK-specific inhibitor, reduced PDT-induced JNK activation as well as caspase-3 activation, indicating that JNK activity is required for PDT-induced caspase activation. Collectively, these results demonstrate that singlet oxygen triggers JNK activation, cytochrome c release, caspase activation and subsequent apoptotic biochemical changes during PDT and show that curcumin is a potent inhibitor for this process.
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Key words: curcumin; PDT; apoptosis; JNK; singlet oxygen

Photodynamic treatment (PDT) of cells involves selective delivery of a photosensitive dye into target cells, followed by visible light irradiation. Interaction of the excited photosensitizer with molecular oxygen results in the formation of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) and hydroxyl radicals, which can damage cellular constituents and are believed responsible for triggering cell destruction [Girotti, 1990; Henderson and Dougherty, 1992]. Photosensitizers include dyes such as Rose Bengal (RB) and methylene blue (MB), drugs such as tetracyclines and chlorpromazine, and endogenous porphyrins [Halliwell and Gutteridge, 1989; Johnson and Ferguson, 1990]. Recently, PDT has been applied to treat solid malignancies, nonmalignant tumors and lung cancer [Pass, 1993; Dougherty et al., 1998].

PDT can induce a diverse range of cellular responses, including apoptosis [Agarwal et al., 1991]. Apoptosis, or programmed cell death, plays an important role in embryogenesis and homeostasis of multicellular organisms. Impairment of the process may cause several human diseases, including neurodegenerative disorders and cancer [Thompson, 1995]. Apoptotic cell death is morphologically characterized by chromatin condensation, membrane blebbing, and cell fragmentation [Kerr et al., 1972]. These morphological changes are also accompanied by biochemical changes including DNA fragmentation. [Wyllie, 1980]. A growing body of

Abbreviations used: PDT, photodynamic treatment; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PAK2, $p21^{Cdc42/Rac}$-activated kinase 2; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species; PARP, Poly (ADP-ribose) polymerase.

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evidence has shown that caspases, a family of cysteine proteases identified in recent years, play a crucial role in apoptotic execution [Martins and Earnshaw, 1997; Nicholson and Thornberry, 1997]. Caspases exist as proenzymes and are activated during apoptosis via proteolytic processing into two smaller subunits, which then form functionally active proteases that can act on many substrates. Among the caspases, caspase-3 (also known as CPP32/Yama/apopain) has been implicated as a downstream effector protease that can be activated by other upstream caspases [Fernandes-Alnemri et al., 1994; Enari et al., 1996]. Caspase-3 can be activated in many cell types by a variety of apoptotic stimuli, and activated caspases can cleave many proteins and enzymes with important roles in a variety of cell functions [Martins and Earnshaw, 1997; Nicholson and Thornberry, 1997].

Curcumin, a common dietary pigment and spice, is used as a traditional Indian medicine [Nadkarni, 1976]. The medicinal roles of curcumin have been demonstrated in wound healing, liver ailments, hepatitis, urinary tract disease and as a cosmetic compound [Kuttan et al., 1985]. Curcumin exhibits a wide range of pharmacological effects, including anti-inflammatory, anti-carcinogenic, hypocholesterolemic and anti-infection activities [Barthelemy et al., 1998; Ramirez-Tortosa et al., 1999; Ramsewak et al., 2000]. As a potent anti-oxidant, curcumin has also been shown to display anti-proliferative and anti-carcinogenic properties in a wide variety of cell lines and animals [Huang et al., 1994; Jiang et al., 1996; Jee et al., 1998; Mahmoud et al., 2000]. Recent studies have shown that the anti-tumor activity of curcumin is attributed to its ability to induce apoptosis via caspase-3 activation [Khar et al., 1999]. Although multiple biological functions of curcumin have been identified, the precise molecular mechanisms underlying its actions appear unknown.

Recently, protein phosphorylation has been shown to be involved in regulating apoptosis. Alteration in activities of several protein kinases can be observed during apoptosis in a variety of cell types [Anderson, 1997]. It appears that c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) can serve as a key component in regulating entry into apoptosis in several cell types under certain circumstances [Xia et al., 1995; Verheij et al., 1996; Seimiya et al., 1997]. JNK/SAPK, an emerging member of the mitogen-activated protein kinase (MAPK) family, can be rapidly activated by environmental stress and inflammatory cytokines [Derijard et al., 1994; Kyriakis et al., 1994], which can also induce ROS generation in target cells [Kyriakis and Avruch, 1996; Anderson, 1997]. Activation of JNK by UV irradiation can be prevented by the anti-oxidant *N*-acetylcysteine [Alder et al., 1995; Tao et al., 1996], suggesting that JNK is activated by oxidative stress. The components in the JNK/SAPK regulation pathway have been identified to be MAPK kinase kinase 1 (MEKK1), SAPK/extracellular-signal-regulated kinase kinase 1 (SEK1), JNK/SAPK, and c-Jun [Sanchez et al., 1994; Yan et al., 1994; Derijard et al., 1995; Lin et al., 1995]. Using dominant, interfering mutants of c-Jun or SEK-1 as tools, activation of the JNK/SAPK pathway has been demonstrated to be required for stress-induced apoptosis in U937 leukemia cells and growth factor withdrawal-triggered apoptosis in PC-12 pheochromocytoma cells [Xia et al., 1995; Verheij et al., 1996]. However, in contrast, two recent reports showed that activation of JNK/SAPK was not essential for Fas-mediated apoptosis in Jurkat T cells and for dexamethasone-induced apoptosis in multiple myeloma cells [Lee et al., 1997; Rudel and Bokoch, 1997]. Hence, the role of JNK/SAPK in apoptosis signaling remains to be better understood.

Previously, we showed that PDT activated caspase-3 and subsequent p21-activated kinase (PAK2) cleavage/activation and DNA fragmentation [Chan et al., 2000]. PAK2, a member of the PAK family of serine/threonine kinases, can bind to and be activated by the small (21 kDa) guanosine triphosphatases (GTPases) Cdc42 and Rac, which regulate actin polymerization [Sells and Chernoff, 1997]. After removal of the PAK2 N-terminal regulatory region by caspase-mediated cleavage, the activity of the C-terminal catalytic fragment (36 kDa) was easily detected by in-gel kinase assays [Tang et al., 1998].

In this study, we describe the effects of curcumin on PDT-induced apoptosis. We report that curcumin inhibited apoptotic biochemical changes such as JNK activation, cytochrome c release, caspase-3 activation, and PAK2 cleavage/activation in A431 cells. We also found that inhibition of JNK activation by SP600125 (a specific JNK inhibitor) prevented PDT-

induced caspase-3 activation. The fact that the formation of ROS during PDT was significantly attenuated by curcumin may account for the inhibitory effects of curcumin on the apoptotic signal cascade elicited by PDT.

EXPERIMENTAL PROCEDURES

Materials

Polyvinylidene fluoride (PVDF) membrane was from Millipore (Bedford, MA). Dulbecco's modified Eagle's medium (DMEM), curcumin, 2′,7′-dichlorofluorescin diacetate (DCF-DA), goat anti-rabbit, and anti-mouse immunoglobulin G (IgG) antibodies conjugated with alkaline phosphatase were from Sigma (St. Louis, MO). Anti-JNK1 (C17), anti-p-JNK (G-7), and anti-α-PAK (C19) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PARP antibody was from Cell Signaling Technology (Beverly, MA). Monoclonal anti-cytochrome c antibody (6H2.B4) was from Imgenex (San Diego, CA). Anthra [1,9-cd] pyrazol-6(2H)-one (SP600125) was from Biomol (Polymouth Meeting, PA). Z-DEVD-AFC was from Calbiochem (La Jolla, CA). CDP-Star™ chemiluminescent substrate for alkaline phosphatase was from Boehringer Mannheim (Mannheim, Germany).

Cell Culture and PDT

Epidermal carcinoma A431 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml rtreptomycin. Cells were plated on 60 mm culture dishes and PDT experiments were performed the following day. For PDT, cells were incubated in media containing various concentrations of RB in the dark for 30 min at 37°C, after which they were irradiated with a commercially available 120 W lamp from a fixed distance of 30 cm for 30 min, and then incubated in the absence of light at 37°C in a CO_2 incubator for the indicated time periods. Cells were then washed twice with ice-cold PBS and lysed on ice for 10 min in 400 μl lysis buffer (20 mM Tris-HCl at pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 20 μM sodium pyrophosphate, and 1 mM sodium orthovanadate). Cell lysates were sonicated on ice for 3×10 s, then centrifuged at 15,000*g* for 20 min at 4°C, and the supernatants used as cell extracts.

Immunoblots

Immunoblotting was carried out essentially as described in previous reports (Chan et al., 2000). Proteins were transferred from SDS–PAGE gels to PVDF membranes and then probed with antibodies against α-PAK(C19) (0.2 μg/ml), JNK1(C17) (0.2 μg/ml), Poly (ADP-ribose) polymerase (PARP) (0.2 μg/ml), or p-JNK (0.2 μg/ml). The proteins of interest were detected using goat anti-rabbit or anti-mouse IgG antibody conjugated with alkaline phosphatase and CDP-Star™, according to the manufacturer's protocol.

Apoptosis Assay

Oligonucleosomal DNA fragmentation in apoptotic cells was measured using the Cell Death Detection ELISA^{plus} kit according to the manufacturer's protocol (Roche Molecular Biochemicals, Mannheim, Germany). Cells (1×10^5) were treated with or without PDT for 30 min at 37°C. Spectrophotometric data were obtained using an ELISA reader at a wavelength of 405 nm.

Caspase-3 Activity Assay

Caspase-3 activity was measured using the fluorogenic substrate Z-DEVD-AFC. Cell lysates (100 μg) were incubated in 250 μl caspase assay buffer (25 mM HEPES at pH 7.5, 0.1% CHAPS, 10 mM dithiothreitol, 100 U/ml aprotinin) containing 0.1 mM Z-DEVD-AFC for 3 h at 37°C. Ice-cold caspase assay buffer (1.25 ml) was then added to the mixture and the relative caspase-3 activity determined using a fluorescence spectrophotometer (Hitachi, F-2000; excitation 400 nm, emission 505 nm).

ROS Assay

ROS arbitrary units were measured using dichlorodihydrofluorescein diacetate (DCFDA) dye. Cells (1.0×10^6) were incubated in 50 μl PBS containing 20 mM DCFDA for 1 h at 37°C, and relative ROS units determined using a fluorescence ELISA reader (excitation 530 nm, emission 485 nm). An aliquot of the cell suspension was lysed and protein concentration determined. Results are expressed as arbitrary absorbance units/mg protein.

Cytochrome c Release Assay

Mitochondrial cytochrome c release assays were performed essentially according to the method described by Yang et al. [1997]. A431

cells (1×10^7) either untreated or PDT-treated were harvested by centrifugation at 800*g* at 4°C for 15 min. After washing three times with ice-cold PBS, cell pellets were resuspended in HEPES-buffer (20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, pH 7.5) containing 250 mM sucrose, homogenized, and centrifuged at 800*g* at 4°C for 15 min. Supernatants were centrifuged at 10,000*g* for 15 min at 4°C, and the mitochondrial pellets were dissolved in SDS sample buffer, subjected to 15% SDS–PAGE, and analyzed by immunoblotting with a monoclonal antibody against cytochrome c.

Statistics

Data were analyzed using one-way ANOVA and the difference between 'with or without' curcumin was evaluated using a two tailed Student's *t*-test and analysis of variance. $P < 0.05$ was considered significant.

Analytical Methods

Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL). SDS–PAGE was performed according to the method of Laemmli [1970] using 10 or 15% acrylamide gels.

RESULTS

Inhibition of PDT-Induced Cell Death and Apoptotic Biochemical Changes in A431 Cells by Curcumin

We examined the effect of curcumin on PDT-induced cell death. A431 cells were incubated with various doses of curcumin, were exposed to PDT or were exposed to PDT after pre-incubation with curcumin, after which cell viability was determined. Curcumin alone up to 50 μM had no effect on viability. Approximately 60% of cells died after PDT and curcumin inhibited this cell death in a dose-dependent manner (Fig. 1A). Further experiments showed a PDT time-dependent decrease in viable cells but a significant increase in the number of viable cells by curcumin pretreatment (Fig. 1B). We, therefore, investigated whether prevention of PDT-induced cell death by curcumin was due to the inhibition of apoptosis by analyzing the effect of curcumin on biochemical events that occur during PDT-induced apoptosis, namely DNA fragmentation, caspase-3 activation, and cleavage of PARP and PAK2 [Chan et al., 2000]. An ELISA was used to quantitatively determine the amount of histone-associated oligonucleosome DNA fragments. We found that in comparison with the untreated cells, PDT induced a 6.5-fold increase in this apoptosis-associated parameter, but in the presence of curcumin this increase was only 1.7-fold (Fig. 2A). We examined PDT-stimulated activation or cleavage of casapse-3, PARP, and PAK2 and found that the concentration of curcumin higher than 100 μM completely blocked the effect of PDT (Fig. 2B–D). Taken together, the results demonstrate that curcumin is a potent inhibitor of PDT-induced apoptosis.

Curcumin and singlet oxygen scavengers prevent PDT-induced ROS formation and subsequent apoptotic biochemical changes in A431 cells—Singlet oxygen ($^1O_2$) has been reported to be an important cellular mediator for PDT-induced responses [Basu-Modak and Tyrrell, 1993; Tao et al., 1996; Klotz et al., 1997]. Our previous data demonstrate that singlet oxygen mediates PDT-induced activation of caspase-3, and subsequent apoptotic biochemical changes in A431 cells [Chan et al., 2000]. To further investigate the molecular mechanisms by which curcumin prevents PDT-induced apoptosis in A431 cells, we tested whether curcumin is a singlet oxygen scavenger. A431 cells were treated with and without L-histidine or α-tocopherol (two well known singlet oxygen scavengers) prior to RB loading and visible light irradiation. The effects of these two compounds on PDT-induced apoptotic biochemical changes was determined. It was found that both $^1O_2$ scavengers potently inhibited PDT-induced cleavage of PARP and PAK2 (Fig. 3A,B). In addition, we examined the effect of curcumin and singlet oxygen scavengers (L-histidine or α-tocopherol) on ROS formation in PDT-treated cells using DCF-DA as the detection reagent. Pretreatment of cells with curcumin, L-histidine or α-tocopherol were significantly attenuated in PDT-stimulated intracellular ROS levels (Fig. 4). Interestingly, mannitol (hydroxyl radical scanvanger) also attenuated ROS formation but did not prevent apoptotic changes by PDT [Fig. 4 and review Chan et al., 2000]. These results indicate curcumin prevents PDT-induced apoptotic biochemical changes by its ability to act as a singlet oxygen scavenger.

Curcumin Inhibits PDT-Induced Mitochondrial Cytochrome c Release

Mitochondrial membrane potential change and cytochrome C release are directly asso-

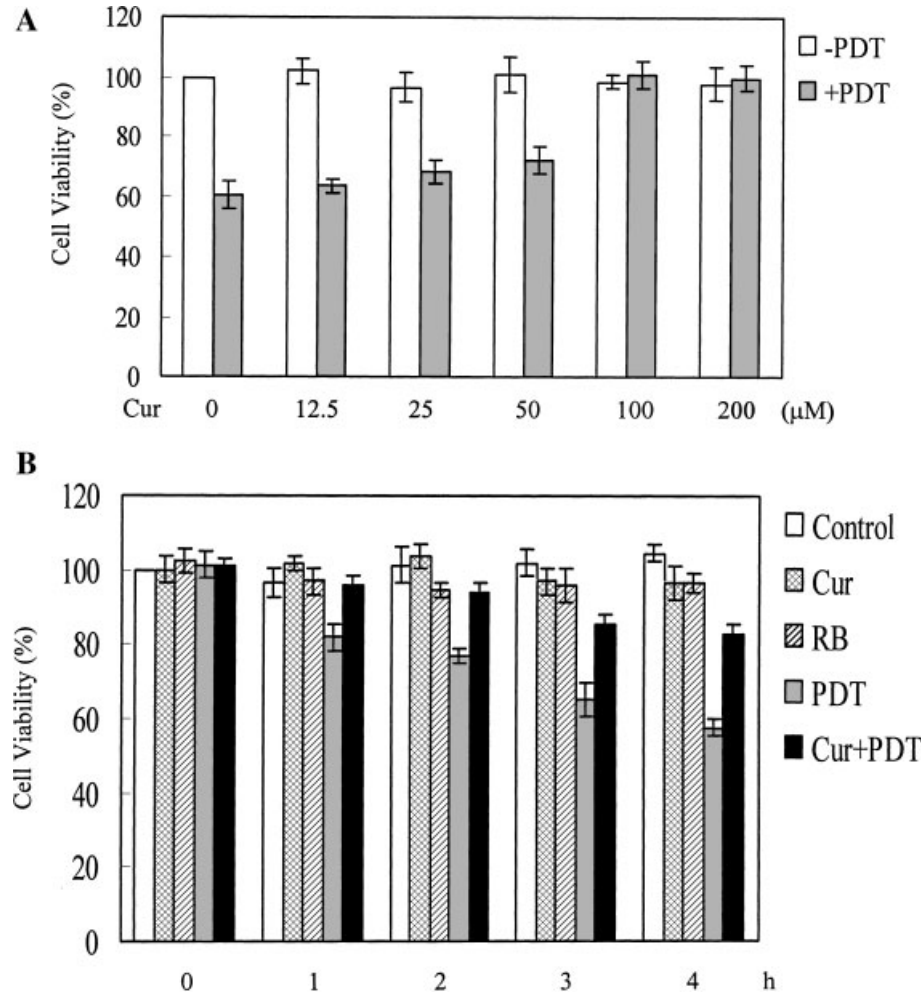
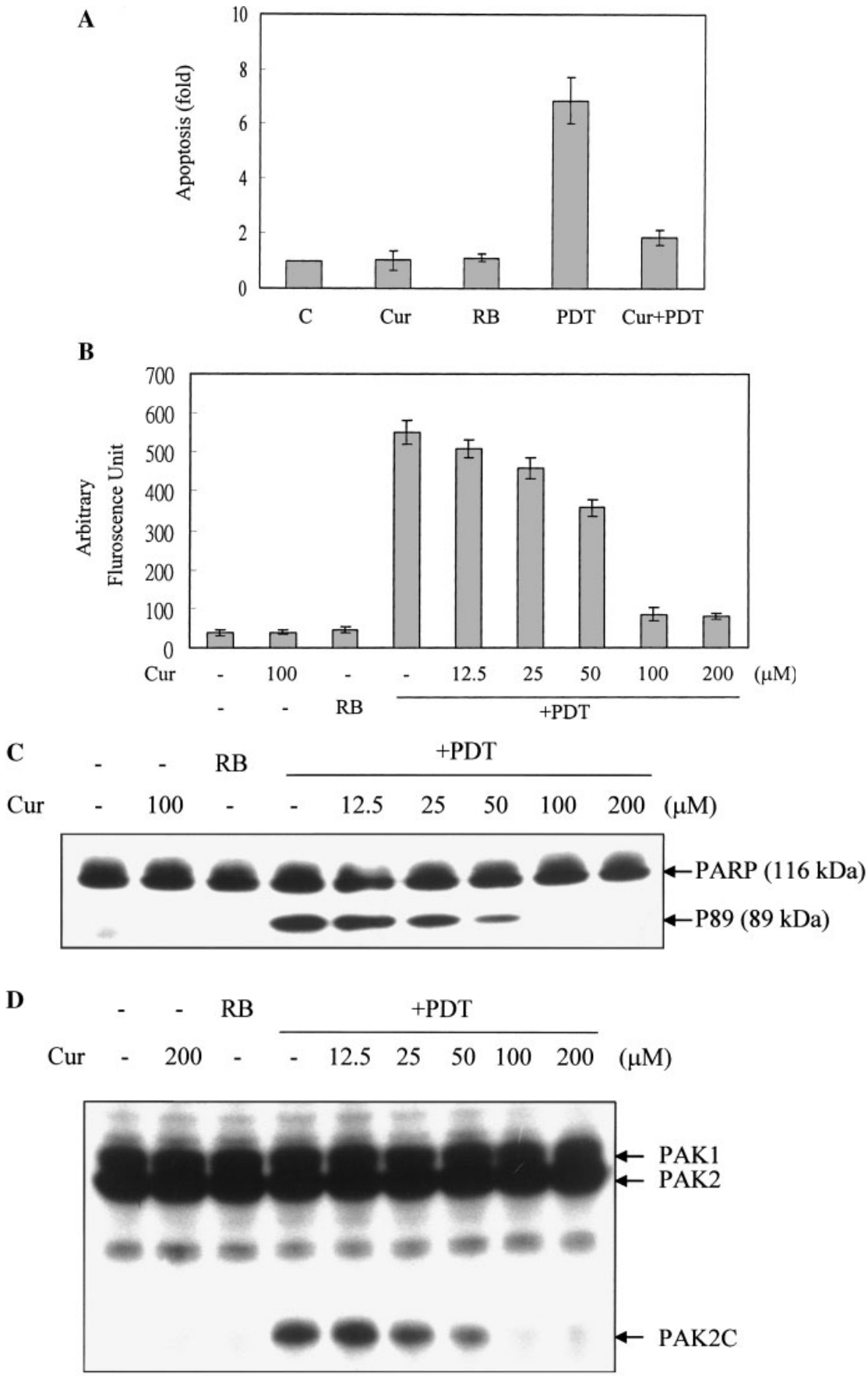


Fig. 1. Curcumin prevents PDT-induced cell death. **A**: A431 cells were incubated with or without various concentrations curcumin (Cur) or Rose Bengal (RB) in the dark at 37°C for 1 h. Cells were then treated with or without visible light for 30 min, and then incubated in the absence of light at 37°C for a further 2 h. Cells were then detached by trypsin digestion, and viable cells counted using microscopy after trypan blue staining. **B**: A431 cells preincubated with or without 100 μM curcumin for 1 h were either untreated or PDT-treated and viable cells counted as in (A) at various time points after PDT, as indicated. The results shown are averages of three independent experiments and standard deviations are all <5%. The number of viable cells in control samples was given the value of 100%.

ciated with apoptosis [Li et al., 1997; Zou et al., 1997; Weber et al., 2003], and both events are observed in PDT-treated cells [Chiu and Oleinick, 2001]. We investigated the effect of curcumin on mitochondrial cytochrome c release. For analysis of cytochrome c release, cytosolic fractions from A431 cells were isolated, and cytochrome c levels were determined by

Fig. 2. Effect of curcumin on PDT-induced apoptosis, activation of caspase-3 and cleavage of PARP and PAK2. **A**: A431 cells were preincubated with 100 μM curcumin (Cur) at 37°C for 1 h. Cells were then subjected to PDT (5 μM RB) and then incubated in the absence of light at 37°C for 2 h, after which cell extracts were prepared. Apoptosis was evaluated using the Cell Death Detection ELISA kit. **B**–**D**: A431 cells were preincubated with various concentrations of curcumin (Cur) at 37°C for 1 h. Cells were then subjected to PDT (5 μM RB) and then incubated in the absence of light at 37°C for 2 h. Cell extracts (60 μg) were analyzed for caspase-3 activity using Z-DEVD-AFC as the substrate (B). Cell extracts (40 μg) were separated by 10% SDS–PAGE and immunoblotted using anti-PARP antibody (C), or anti-αPAK(C19) antibody (D). PAK2C represents the C-terminal cleavage product of PAK2. P89 (89 kDa) represents the cleavage product of PARP. The data are representative of three independent experiments.


A
Apoptosis (fold)
10
8
6
4
2
0
C
Cur
RB
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B
Arbitrary Fluroscence Unit
700
600
500
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200
100
0
Cur - 100 - - 12.5 25 50 100 200 (μM)
- - RB +PDT
C
- - RB +PDT
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PARP (116 kDa)
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PAK1
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PAK2C

Fig. 2.

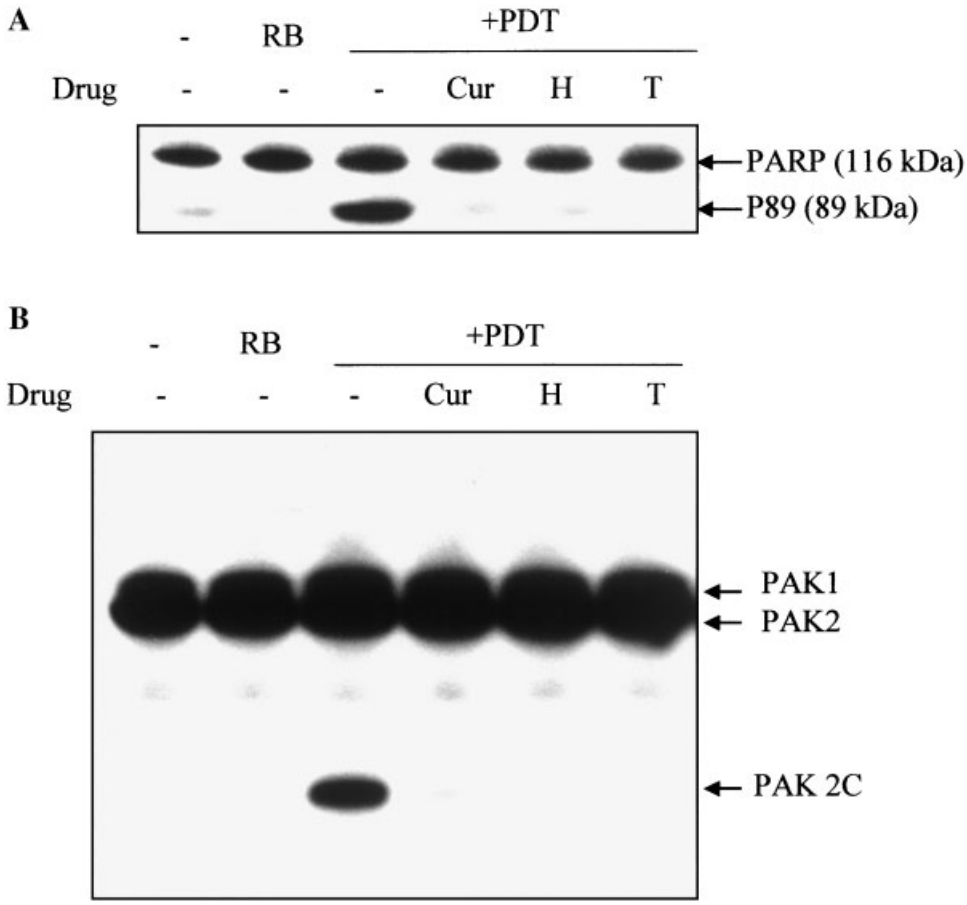

Fig. 3. Effect of curcumin and singlet oxygen scavengers on PDT-induced cleavage of PARP and PAK2. A431 cells were incubated with curcumin (100 μM; Cur) or L-histidine (1 mM; H) or α-tocopherol (300 μM; T) at 37°C for 1 h and then subjected to PDT (5 μM RB) followed by incubation in the absence of light at 37°C for another 2 h. Cell extracts were prepared and analyzed for cleavage of PARP (**A**) and cleavage of PAK2 (**B**), as described in the legend for Figure 2. PAK2C represents the C-terminal cleavage product of PAK2. P89 (89 kDa) represents the cleavage product of PARP. The data are representative of three independent experiments.

immunoblotting. We found that PDT caused significant cytochrome c release into the cytosol compared with non-irradiated control cells (Fig. 5). Curcumin pretreatment dose-dependently inhibited this cytochrome c release (Fig. 5).

Curcumin Inhibits JNK Activation Required for Caspase-3 Activation During PDT-Induced Apoptosis

To determine the relationship between JNK and caspase-3 activities during PDT-induced apoptosis, we examined the effect of the specific JNK inhibitor SP600125 [Bennett et al., 2001] on PDT-treated A431 cells. We found that SP600125 reduced PDT-stimulated JNK activity to 52% of that in control cells, but had no effect on JNK protein levels (Fig. 6A). We also found that SP600125 inhibition of JNK activity reduced cytochrome c release, caspase-3 activation, PARP cleavage, and PAK2 cleavage (Fig. 6B–D). These findings indicate that JNK activity is required for caspase-3 activation and subsequent apoptotic biochemical changes during PDT-induced apoptosis. When we examined the effect of curcumin on PDT-induced JNK activity we found that curcumin dose-dependently inhibited JNK activity, with 100 μM curcumin pretreatment causing ~83% reduction in activity, but having little effect on JNK protein levels (Fig. 7).

DISCUSSION

In recent years, PDT has emerged as a promising therapeutic protocol for treatment of malignant and non-malignant diseases [Pass,

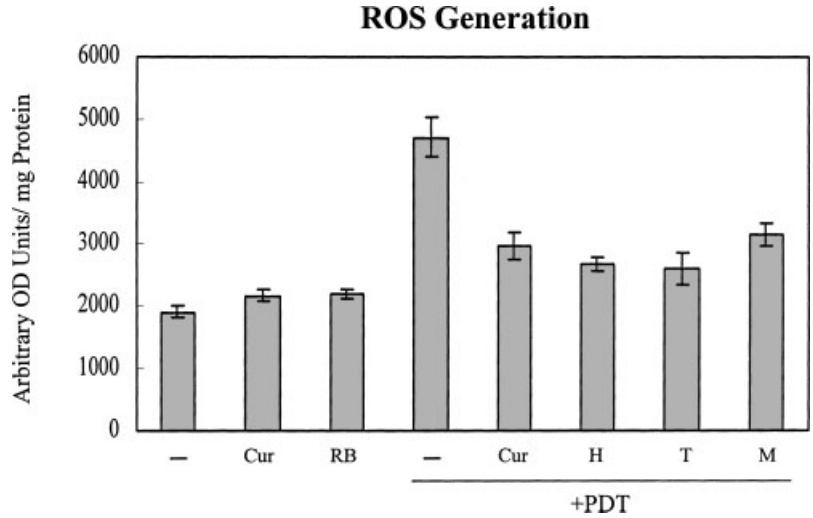

Fig. 4. Curcumin attenuates PDT-induced oxidative stress. A431 cells were incubated with curcumin (100 μM; Cur) or L-histidine (1 mM; H) or α-tocopherol (300 μM; T), or mannitol (5 mM; M) at 37°C for 1 h and then subjected to PDT (5 μM RB) followed by incubation in the absence of light at 37°C for another 2 h. The generation of ROS was assayed using dichlorodihydrofluorescein diacetate (DCF-DA) and is expressed as absorbance/mg of protein. The data are representative of three independent experiments.

1993]. Recently, we have shown that PDT elicits singlet oxygen formation in A431 cells and singlet oxygen scavengers prevent PDT-induced caspase-3 cleavage/activation and subsequent PAK2 activation [Chan et al., 2000]. We also demonstrate that curcumin can prevent UV irradiation-induced apoptotic changes, including JNK activation, loss of mitochondrial membrane potential, mitochondrial release of cytochrome C, caspase-3 activation, and cleavage/activation of PAK2 in A431 cells. Flow cytometric analysis using the cell permeable dye DCF-DA as an indicator of ROS generation revealed that increase in intracellular oxidative stress caused by UV irradiation could be abolished by curcumin [Chan et al., 2003]. In the present study, we show that curcumin at doses higher than 100 μM inhibits PDT-induced, caspase-dependent apoptotic biochemical changes in A431 cells (Fig. 1). Others studies have shown that curcumin can both stimulate and inhibit apoptotic signaling. For instance, curcumin induced apoptosis in human melanoma cells (30–60 μM for 24 h) [Bush et al., 2001], human leukemia HL 60 cells (10–40 μM for 16–24 h) [Kuo et al., 1996; Anto et al., 2002], AK-5 tumor cells (10 μM for 18 h) [Bhaumik et al., 1999; Khar et al., 1999], and MCF-7 breast cancer cells (25 μM for 24 h) [Choudhuri et al., 2002]. In contrast, both dexamethane-induced apoptosis in rat thymocytes and chemotherapy-induced apoptosis in breast cancer cells were

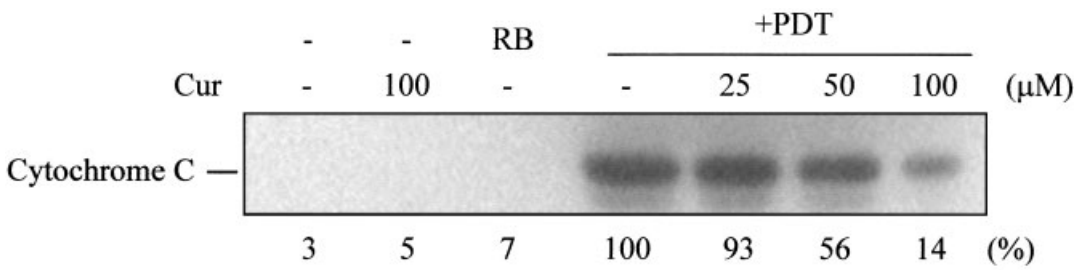

Fig. 5. Effect of curcumin on PDT-induced cytochrome c release from mitochondria. A431 cells were preincubated with various concentrations of curcumin (Cur) at 37°C for 1 h and then exposed to PDT. Cytosolic and mitochondrial fractions were separated, and cytosol aliquots (60 μg) were resolved by 15% SDS–PAGE and then immunoblotted using anti-cytochrome c antibody. The data are representative of three independent experiments.

inhibited by curcumin (10 μM for 12 h) [Jaruga et al., 1998; Somasundaram et al., 2002]. We previously also showed that curcumin significantly attenuates UV irradiation-induced ROS formation and apoptosis in epidermoid carcinoma A431 cells (25–50 μM for 2–3 h) [Chan et al., 2003]. In the present report, our results show that curcumin at doses greater than 100 μM blocks PDT–induced apoptotic biochemical changes in A431 cells when we use 5 μM RB as the photosensitizer in PDT (Fig. 2). We also found that 10 μM curcumin pretreatment could effectively inhibit PDT-induced cell apoptosis in A431 cells during using 1 μM RB as the

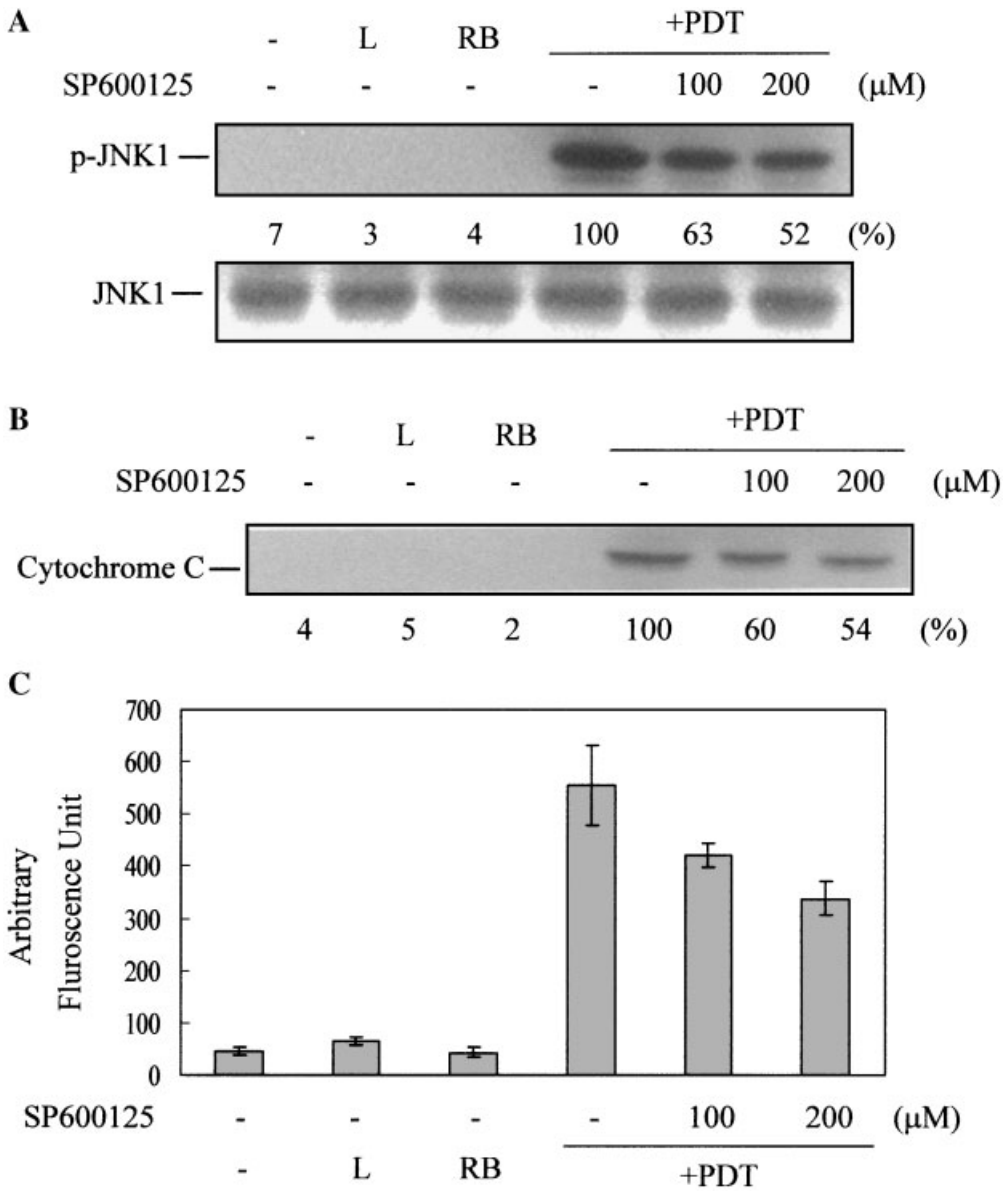

Fig. 6. Effect of the JNK-specific inhibitor SP600125 on PDT-induced cytochrome c release, caspase-3 activation and cleavage of PARP and PAK2. A431 cells were preincubated with various concentrations of SP600125 at 37°C for 1 h and then exposed to PDT. **A**: Cell extracts (60μg) prepared 15 min after PDT were separated by 12.5% SDS–PAGE and then immunoblotted with anti-p-JNK antibody. The **lower panel** shows an immunoblot of JNK1 protein from 60 μg cell extract. **B**: Cell extracts (60 μg) prepared 2 h after PDT were separated by 15% SDS–PAGE and then immunoblotted with anti-cytochrome c antibody. **C**: Cell extracts (60 μg) prepared 2 h after PDT were analyzed for caspase-3 activity using Z-DEVD-AFC as the substrate. **D**, **E**: Cell extracts (40 μg) prepared 2 h after PDT were resolved by 10% SDS–PAGE and analyzed for cleavage of PARP (D) and cleavage of PAK2 (E), as described in the legend for Figure 2. PAK2C represents the C-terminal cleavage product of PAK2. P89 (89 kDa) represents the cleavage product of PARP. The data are representative of three independent experiments. L, visible light.

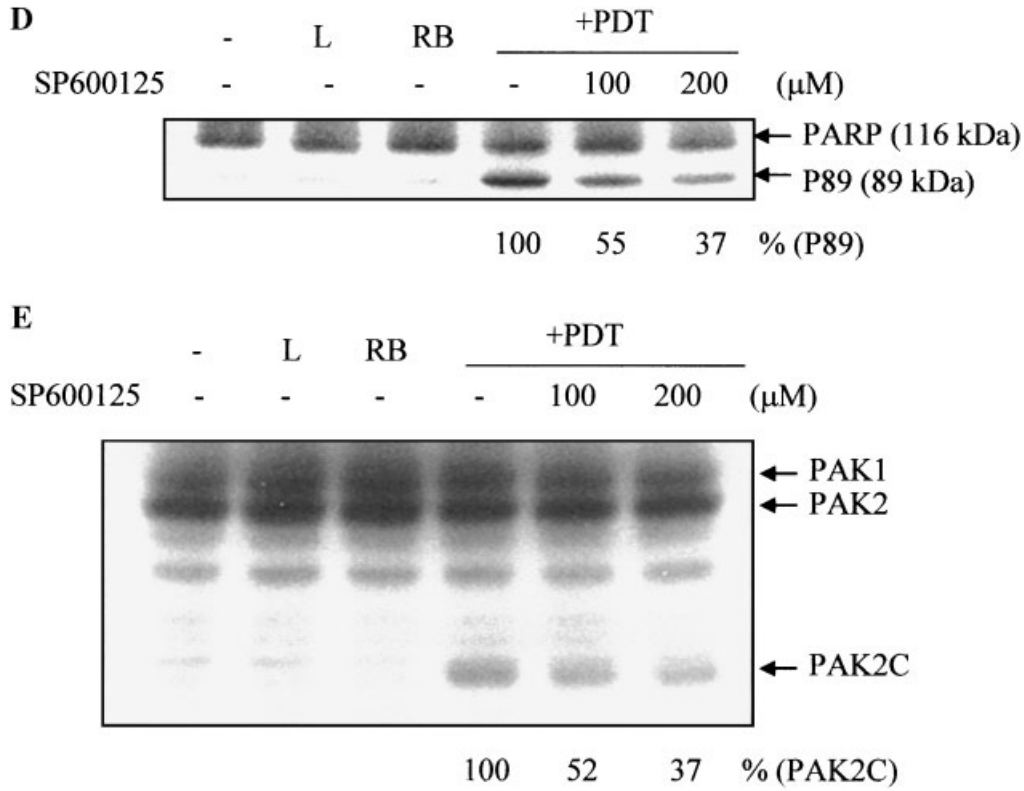

Fig. 6. (*Continued*)

photosensitizer (data not shown). The effect of PDT-induced apoptotic biochemical changes in A431 cells was dependent on RB concentration. Pretreatment of 5 μM RB induced stronger apoptotic biochemical changes than 1 μM during PDT in A431 cells [Chan et al., 2000]. Our results further demonstrated that severe apoptotical biochemical changes induced by 5 μM RB in PDT could be effectively blocked by 100 μM curcumin. It indicated strongly that curcumin is a potent inhibitor for the effect of PDT-induced apoptosis. These results also demonstrate the effective dosage of curcumin to prevent PDT-induced apoptosis is dependent on photosensitizer concentration. Taken together these observations and our research results seem to implicate that there is some cell type specificity and treatment protocol (i.e., treatment period

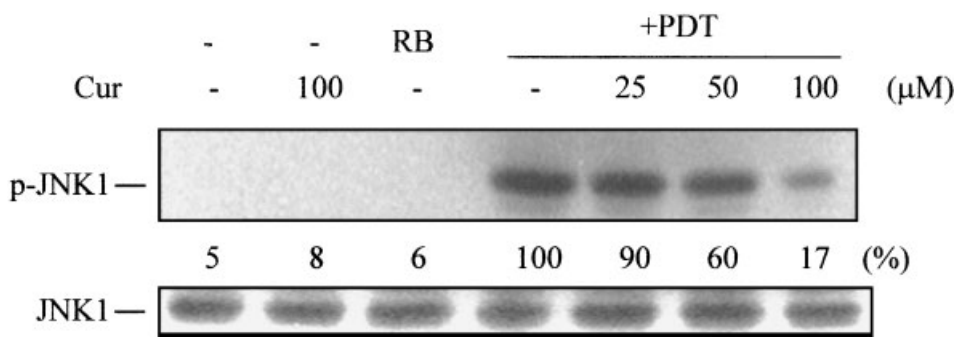

Fig. 7. Effect of curcumin on PDT-induced JNK activation. A431 cells were preincubated with various concentrations of curcumin (Cur) at 37°C for 1 h, and then exposed to PDT. Cell extracts (60 μg) were prepared 15 min later, electroblotted onto PVDF membranes and probed with anti-p-JNK antibody. The signal intensity on autoradiographs was measured using a densitometer, and the maximum signal was given the value of 100%. The **lower panel** shows immunoblot analysis of JNK1 protein from 60 μg cell extract at each dose.

and dosage of curcumin) may determine the effect of curcumin. However, the molecular mechanisms of curcumin on cell apoptosis need further investigation for regimen employed.

The inhibitory effect of curcumin on apoptotic biochemical changes triggered by several stimuli has been attributed to its anti-oxidant properties [Ruby et al., 1995]. Oxidative stress is recognized as a stimulator of cell responses such as apoptosis. Not only can direct exposure of cells to oxidative stress induce apoptosis, but anti-oxidants also protect cells against apoptosis induced by various stimuli that do not exert direct oxidant effects [Buttke and Sandstrom, 1994; Slater et al., 1995]. Mechanisms underlying the anti-oxidant and anti-inflammatory properties of curcumin may involve glutathione (GSH)-linked detoxification. Curcumin treatment can increase non-protein sulfhydryls (NPSH), a surrogate for GSH, in a dose-dependent fashion. This increase may be due to increased γ-glutamyl cysteine synthetase (γ-GCS) activity, which catalyzes GSH biosynthesis [Piper et al., 1998]. Recently, it was found that curcumin at very low concentrations inhibited the singlet oxygen-dependent formation of 2,2,6,6-tetramethypiperidine *N*-oxyl (TEMPO), showing curcumin is able to effectively quench singlet oxygen [Das and Das, 2002]. Since PDT can also generate hydroxyl radicals ($^{\bullet}OH$) in addition to $^1O_2$, and that most $^1O_2$ scavengers are good $^{\bullet}OH$ quenchers [Basu-Modak and Tyrrell, 1993], it may be that $^{\bullet}OH$ quenching contributes to the anti-apoptotic effects of curcumin, L-histidine and α-tocopherol on PDT-induced biochemical changes. However, this possibility can be ruled out by the observation that mannitol, a known $^{\bullet}OH$ scavenger which quenches $^{\bullet}OH$ far more specifically due to its low rate constants for reaction with $^1O_2$ [Farhataziz and Ross, 1977; Wilkinson and Brummer, 1981], had no effect on PDT-induced apoptotic biochemical changes [Chan et al., 2000]. Our finding that curcumin attenuated PDT-induced intracellular ROS formation supports the notion that curcumin suppresses apoptosis by quenching singlet oxygen that forms following PDT (Figs. 2 and 3).

It appears that mitochondria act as important conduits for signals during programmed cell death, and that loss of mitochondrial integrity can be promoted or inhibited by many key regulators of apoptosis [Kroemer et al., 1997; Green and Reed, 1998]. For instance, diverse cellular stresses including heat shock, DNA damage and oxidative stress, result in caspase activation through cytochrome c release from the mitochondrial intermembrane space into the cytoplasm [Liu et al., 1996; Green and Reed, 1998]. To further elucidate mechanisms underlying curcumin inhibition of PDT-induced apoptosis, we examined mitochondrial release of cytochrome c. We found that PDT-induced mitochondrial release of cytochrome c was inhibited in a dose-dependent manner by curcumin (Fig. 5).

JNK plays roles in many cell responses, including entry into apoptosis. While it has been reported that curcumin can block JNK activation by various agents or treatments [Ruby et al., 1995; Singh and Aggarwal, 1995; Chen and Tan, 1998], the mechanisms by which it operates remain unclear. Using SP600125, an inhibitor specific for JNK, we demonstrated that PDT-induced caspase-3 activation in A431 cells was mediated by JNK activity (Fig. 6). These observations taken together with the findings that singlet oxygen generation and JNK activation triggered by PDT can be blocked by curcumin, support the hypothesis that curcumin inhibits PDT-induced apoptotic biochemical changes by blocking singlet oxygen formation and JNK activation, both of which are important triggers for cytochrome c release from mitochondria into the cytoplasm and subsequent activation of caspases. A model for PDT-triggered apoptotic signal pathway is proposed as following: PDT → singlet oxygen ($^1O_2$) generation → JNK activation → cytochrome c release → caspase-3 activation → PAK2 cleavage/activation.

Curcumin prevent chemotherapy treatment induced apoptosis by inhibition of ROS gereration and JNK activation in MCF-7, MDA-MB-231, and BT-474 human breast cancer cell [Somasundaram et al., 2002]. The in vivo model also demonstrate that dietary curcumin significanty prevent cyclophosphamide-induced breast tumor growth inhibition. In addition, Jaruga et al. [1998] have demonstrated that the absorption rate of curcumin in intestinal lumen is up to 80% by passive diffusion [Jaruga et al., 1998]. These findings highlight the need for further study into the mechanisms of curcumin action, especially in relation to the possible use of dietary curcumin in patients with breast cancer chemotherapy or receiving PDT.

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