Oxidative Stress Induced by Curcumin Promotes the Death of Cutaneous T-cell Lymphoma (HuT-78) by Disrupting the Function of Several Molecular Targets

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Abstract

Curcumin is known to exert its anticancer effect either by scavenging or by generating reactive oxygen species (ROS). In this study, we report that curcumin-mediated rapid generation of ROS induces apoptosis by modulating different cell survival and cell death pathways in HuT-78 cells. Curcumin induces the activation of caspase-8, -2, and -9, alteration of mitochondrial membrane potential, release of cytochrome c, and activation of caspase-3 and concomitant PARP cleavage, but the addition of caspase inhibitors only partially blocked the curcumin-mediated apoptosis. Curcumin also downregulates the expression of antiapoptotic proteins c-FLIP, Bcl-xL, cellular inhibitor of apoptosis protein, and X-linked IAP in a ROS-dependent manner. Curcumin disrupts the integrity of IKK and beclin-1 by degrading Hsp90. Degradation of IKK leads to the inhibition of constitutive NF-kB. Degradation of beclin-1 by curcumin leads to the accumulation of autophagy-specific marker, microtubule-associated protein-I light chain 3 (LC3), LC3-I. Our findings indicate that HuT-78 cells are vulnerable to oxidative stress induced by curcumin and as a result eventually undergo cell death.

Introduction

Cutaneous T-cell lymphomas (CTCL) are a class of non-Hodgkin lymphomas characterized by the uncontrolled growth of mature CD4+ T cells with distinct T-cell markers. The 2 main forms are mycosis fungoides and its leukemic counterpart Sézary syndrome (1, 2). Defective T-cell apoptosis and constitutive activation of NF-kB contribute to the pathogenesis of CTCLs (2, 3). NF-kB is a regulator of many genes involved in inflammation, cell proliferation, and apoptosis. Activation of NF-kB enhances the expression of antiapoptotic proteins such as inhibitor of apoptosis proteins (IAP), X-linked IAP (XIAP), Bcl-2, Bcl-xL, and c-FLIP (4). Human IAP proteins, including cIAP-1, cIAP-2, XIAP, are known to inhibit apoptosis by inhibiting effector caspases, caspase-7 and -3 (5).

Apoptotic stimulation activates at least 1 of the 2 major apoptotic pathways, the intrinsic or mitochondrial cell death pathway and the extrinsic or receptor-mediated cell death pathway (6). The mitochondrial pathway leads to disruption of mitochondrial membrane, release of cytochrome c into the cytosol, which then binds to the apoptotic protease activating factor complex and triggers the activation of procaspase-9 to active caspase-9 (7). Hsp90 is a molecular chaperone that is crucial for the stability of many client proteins involved in signaling pathways for cell survival (8, 9). It has been shown that Hsp90 function is required for constitutive IKK/NF-kB activity in lymphoma cells (10). It is also reported recently that Hsp90 protects beclin-1 (Atg6), a key autophagy-promoting protein that regulates the formation of autophagosomes from ubiquitination-associated proteolytic degradation (11).

Curcumin, a yellow pigment derived from rhizome of Curcuma longa, has been a subject of extensive investigations over the last 5 decades and has confirmed its antiapoptotic activity in a panel of tumor cells (12). The potent anticancer property of curcumin is credited to its antioxidant effect that restrains free radical–mediated lipid peroxidation and DNA damage (13). However, an increasing number of recent reports showed that curcumin exerts its anticancer effect by acting as a pro-oxidant, by inducing reactive oxygen species (ROS) generation. Interestingly, Kang and colleagues found that the antitumor effect of curcumin was due to its pro-oxidant effect on tumor cells (12, 14).

Recently, it has been reported that curcumin selectively induces apoptosis in CTCL cell lines by inhibiting STAT-3 and NF-kB (15). But this study did not address the antioxidative or pro-oxidative effect of curcumin on CTCL cells. Thus, the present study was designed to find out the effect of curcumin on intracellular ROS formation and its...
potential link with curcumin-induced cytotoxicity in HuT-78 cells.

Here, we report that curcumin through generation of ROS disrupts the function of several important molecules involved in cell survival pathways, and as a consequence, HuT-78 cells undergo apoptosis.

Materials and Methods

Cell culture and reagents

Human CTCL cell line, HuT-78, was procured from National Centre for Cell Science (Pune, India), a cell banking repository that supplies cell lines for research purposes. MyLa was obtained from and authenticated by European Collection of Animal Cell Cultures (Salisbury, UK). Both the cell lines were passaged for less than 3 months before use in this study. These cell lines were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, Gibco (16). Curcumin, N-acetyl-L-cysteine (NAC), rapamycin, 3-methyladenine (3-MA), bocyanine iodide (DiOC6), anti-actin antibody, anti-rabbit horseradish peroxidase antibody, and anti-mouse horseradish peroxidase antibody, and anti-rabbit horseradish peroxidase antibody were purchased from Sigma. Dihydroethidium and Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit were purchased from Molecular Probes (Invitrogen). Annexin V-FITC Apoptosis Detection Kit, APO-BRDU Kit, and antibodies against cytochrome c, PARP, Bcl-xL, cIAP-1, cIAP-2, XIAP, Hsp90, Hsp70, IKK-α, IKK-β, beclin-1, and caspase-2 were purchased from BD PharMingen. LC3, Atg5, and Atg7 antibodies were purchased from Cell Signaling. 17-Allylamino-17-demethoxygeldanamycin (17-AAG) was purchased from Calbiochem.

Measurement of ROS

Dihydroethidium fluorescent probe was used to measure the intracellular generation of superoxide anion radical, O$_2^-$ (17). Briefly, 5 × 10$^5$ cells (HuT-78 or MyLa) were seeded and treated with curcumin (25 μmol/L) at different time points. Cells were exposed to 5 μmol/L dihydroethidium for 30 minutes at 37°C, and the fluorescence intensity in cells was determined by using flow cytometer (Becton Dickinson).

H$_2$O$_2$ release in the culture supernatant of HuT-78 or MyLa cells was measured by Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit according to manufacturer’s instructions. Fluorescence was determined using a Synergy 4 multidetection microplate reader (Bio-Tek) with an excitation of 530 nm and emission at 590 nm.

PI exclusion assay for cell viability

Cell viability was determined by PI exclusion assay (16). Briefly, 2 × 10$^5$ cells (HuT-78 or MyLa) were treated with different concentration of curcumin (0–25 μmol/L) for 24 hours (18). The level of PI incorporation was quantitated by flow cytometry on a FACSCalibur (Becton Dickinson).

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling

To measure the DNA strand breaks during apoptosis, TUNEL assay was conducted using APO-BRDU Kit (BD PharMingen) according to manufacturer’s protocol as described by Gahlot and colleagues (16). Stained cells were analyzed with flow cytometry.

Annexin V staining

Annexin V staining kit (BD PharMingen) was used as described previously (16). Stained cells were analyzed with a FACSCalibur (Becton Dickinson).

Mitochondrial membrane potential

The change of mitochondrial membrane potential (MMP) was determined by the retention of the dye DiOC6 as described by Rishi and colleagues (19).

Caspase-8, -9, and -3 activity assay

Caspase-8 (Sigma), caspase-9 (Caspase-Glo 9 Assay System), and caspase-3 (Promega) activities were determined according to manufacturer’s protocol.

Measurement of cytochrome c release

Cytosolic extracts were prepared according to the method as described by Miyoshi and colleagues (20). The release of cytochrome c into cytosol was measured by immunoblotting.

Western blot analysis

For Western blot analysis, cells lysates were prepared after treatment as described previously (16). Protein extracts were resolved on 10% to 12% SDS-PAGE and analyzed by Western blotting using specific antibodies against caspase-2, Bid, cytochrome c, PARP, c-FLIP, Bcl-xL, cIAP-1, cIAP-2, XIAP, Hsp90, Hsp70, IKK-α, IKK-β, beclin-1, and LC3. Densitometry of individual bands was determined using Scion Image software (Scion Corporation).

Electrophoretic mobility shift assay

NF-κB DNA-binding activity was determined by electrophoretic mobility shift assay (EMSA) as described previously (19).

Statistical analysis

Statistical significance of the differences was determined by the paired 2-tailed Student t test using Microsoft Excel software. P values <0.05 were considered as statistically significant.

Results

Curcumin generates ROS in HuT-78 and MyLa cells

It has been shown that many chemotherapeutic agents induce ROS-mediated killing of cancer cells (21). The role of ROS in curcumin-mediated apoptosis of HuT-78 cells is yet to be explored. To determine whether curcumin
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Curcumin shows cytotoxic activity against HuT-78 and MyLa cells in a ROS-dependent manner

Recently, it has been reported that curcumin is cytotoxic for CTCL cells (15). So, we were interested to see whether ROS is involved in curcumin-mediated killing of HuT-78 and MyLa cells. For this purpose, cells were treated with different concentrations of curcumin (0–25 μmol/L) for 24 hours, and cell viability was assessed by live PI dye exclusion method. Concentration-dependent cytotoxic effects were observed in curcumin-treated both cell lines (Fig. 1D). Curcumin-mediated cell death was found to be ROS-dependent, because the addition of NAC completely suppressed the cytotoxic effect of curcumin. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay of curcumin-treated cells further confirmed that curcumin-mediated killing of HuT-78 cells is ROS-dependent. As shown in Fig. 1E, curcumin treatment showed dose-dependent increase in the percentage of apoptotic cells from 3% to 78%, and pretreatment with NAC markedly reduced the degree of apoptosis. Under similar conditions, NAC (10 mmol/L) alone did not show any effect on cell viability (data not shown).

Curcumin-mediated ROS generation induces caspase activation, alters MMP followed by cytochrome c release

To determine the involvement of caspases in curcumin-mediated apoptosis of HuT-78 cells, we examined the activation of different caspases. First, we measured the activation of caspase-8, the initiator caspase, in curcumin (0–25 μmol/L)-treated HuT-78 cells by colorimetric assay. As shown in Fig. 2A, a dose-dependent increase in caspase-8 activity was obtained when measured after 24 hours of curcumin treatment. Caspase-8 activation was blocked when NAC or broad-range caspase inhibitor, z-VAD-fmk, was present in the system. When the activation of another initiator caspase, caspase-2, was measured by immunoblotting, we obtained dose-dependent activation of caspase-2 in HuT-78 cells after 24-hour treatment with curcumin. Prior treatment of cells either with NAC or different caspase inhibitors (z-VAD-fmk, z-IETD-fmk, and Ac-DEVD-CHO) blocked this activation (Fig. 2B). The cleavage of Bcl-2 family member Bid to t-Bid, a substrate for caspase-8, is involved in linking extrinsic pathway to mitochondrial pathway (6). Therefore, we investigated the cleavage of Bid to t-Bid in HuT-78 cells treated with curcumin. Enhanced cleavage of Bid was observed in the cytosolic extracts of curcumin (25 μmol/L)-treated cells as compared with control cells after 24 hours. The cleavage of Bid was suppressed in the presence of NAC or z-VAD-fmk (Fig. 2C).

Next, we wanted to see whether mitochondrial pathway is involved in curcumin-mediated apoptosis of HuT-78 cells. Cells were treated with different concentrations of curcumin (0–25 μmol/L) for 24 hours and then DiOC6 staining was conducted. As shown in Fig. 2D, a dose-dependent increase in MMP was seen, which indicated mitochondrial hyperpolarization. When 25 μmol/L curcumin-treated cells were analyzed after staining, depolarization peak was clearly visible and this peak disappeared when cells were pretreated with NAC. Alteration of MMP is known to cause release of cytochrome c into the cytosol. Cytochrome c release from mitochondria into the cytosol of curcumin-treated HuT-78 cells was detected by immunoblotting. Pretreatment with NAC blocked the cytochrome c release, but z-VAD-fmk pretreatment could not significantly suppress the release of cytochrome c (Fig. 2E).

Next, we measured the activation of caspase-9 and then the activation of caspase-3 in curcumin-treated HuT-78 cells after 24 hours. Concentration-dependent activation of both caspase-9 and -3 was observed (Fig. 3A and B). Activation of caspase-3 was markedly reduced in the presence of NAC or broad-range caspase inhibitor, z-VAD-fmk (Fig. 3B). PARP cleavage was detected by immunoblotting using anti-PARP antibody. PARP cleavage could be detected in cells treated with 12.5 and 25 μmol/L of curcumin, but at lower concentration, no cleavage was observed. Remarkably, this curcumin-mediated cleavage of PARP was completely reverted in the presence of NAC (Fig. 3C).

We found that curcumin activated initiator (caspase-8, -2, and -9) and executioner caspase, caspase-3, so the next obvious question we asked was that whether caspase-mediated cell death pathway is solely responsible for killing of HuT-78 cells induced by curcumin. For this purpose, the effect of different caspase inhibitors on curcumin-mediated death of HuT-78 cells was studied. Cells were first treated with different caspase inhibitors, namely, z-VAD-fmk, z-IETD-fmk, and Ac-DEVD-CHO for 1 hour and then treated with 25 μmol/L curcumin. After 24 hours, Annexin V staining was conducted to confirm cell death. As shown in Fig. 3D, only partial inhibition (12%–16%) in cell death was observed with

Increases ROS production in HuT-78 cells, first we measured the intracellular ROS generation at different time points in curcumin-treated cells using superoxide anion-sensitive probe dihydroethidium. Our fluorescence-activated cell-sorting (FACS) analysis revealed that intracellular superoxide anion free radical (O2−) level was increased in HuT-78 cells following treatment with 25 μmol/L curcumin compared with untreated control cells. Superoxide anion free radical (O2−) was detected as early as 0.5 hours after treatment with 25 μmol/L curcumin, peaked at 6 hours (Fig. 1B). We also tested the effect of curcumin on O2−-generation in another CTCL cell line, MyLa. Similar results were obtained (Fig. 1B). We also measured extracellular H2O2 in the culture supernatants using Amplex Red Hydrogen Peroxide/Peroxidase assay at similar time points. We found that curcumin treatment resulted in enhanced accumulation of H2O2 in culture supernatant, peaked at 2 hours (Fig. 1C). Prior treatment of cells with antioxidant, NAC (10 mmol/L), markedly blocked curcumin-mediated ROS generation (data not shown).

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Figure 1. Curcumin generates ROS in HuT-78 and MyLa cells and induces cell death. A, chemical structure of curcumin. B, HuT-78 or MyLa cells were treated with 25 μmol/L of curcumin for indicated times, and the level of O$_2^·$ was assayed by dihydroethidium staining using flow cytometer. Data represent the results from 1 of the 3 similar experiments. C, at similar time points as in B, H$_2$O$_2$ accumulation in supernatants was measured using Amplex Red Hydrogen Peroxide Assay Kit. Data represent mean values ± SD of 3 similar experiments. *P < 0.05 vs. control. D, HuT-78 or MyLa cells were treated with curcumin (0–25 μmol/L) in the presence and absence of NAC (10 mmol/L) for 24 h and examined for cell viability by live PI exclusion method, where M1 represents percentage of dead cells. Bar graph represents killing of HuT-78 and MyLa cells. Values are expressed as mean ± SD of 3 similar experiments. *P < 0.05 vs. control. E, HuT-78 cells were treated similarly for 24 hours, and apoptosis was detected by TUNEL assay through flow cytometry at FL-1 channel. The M1 and M2 gates demarcate apoptotic and nonapoptotic populations, respectively.
different caspase inhibitors, whereas prior treatment with NAC significantly blocked cell death induced by curcumin.

**Curcumin downregulates c-FLIP, Bcl-xL, cleaves XIAP, and Hsp90 in HuT-78 cells**

Western blot analysis revealed that curcumin effectively downregulated the expression of both c-FLIP_L and c-FLIP_S as compared with control (Fig. 4A). Curcumin also inhibited Bcl-xL expression in a dose-dependent manner (Fig. 4B). When immunoblotting was conducted to detect the expression of cIAP family proteins, cIAP-1 and cIAP-2, we found that 25 μmol/L curcumin significantly inhibited the expression of cIAP-2 as compared with cIAP-1 (Fig. 4C). The expression of c-FLIP, Bcl-xL, and cIAP-2 appeared to be ROS-dependent, because the addition of NAC suppressed the inhibitory effect of curcumin on these proteins. We also checked the expression of XIAP in cell lysates prepared from curcumin-treated HuT-78 cells after 24 hours. Immunoblotting revealed that curcumin at 25 μmol/L concentration cleaved XIAP. The cleaved fragment of XIAP was not visible in NAC-pretreated cells (Fig. 4D). Furthermore, to determine whether curcumin-induced activation of caspases has any effect on XIAP, we treated HuT-78 cells with 25 μmol/L curcumin in the presence or absence of different inhibitors of caspases. XIAP cleavage was completely blocked in the presence of z-VAD-fmk, a broad-range caspase inhibitor, whereas caspase-8–specific inhibitor, z-IETD-fmk, slightly inhibited the XIAP cleavage. In contrast, caspase-3–specific
HuT-78 cells were treated with various concentrations (0–25 μmol/L) of curcumin for 24 hours, and caspase-9 activity was determined by cell-based luminescence assay. Data represent mean values ± SD of 3 similar experiments. *P < 0.05 vs. control. B, cells were treated with curcumin (0–25 μmol/L) in the presence or absence of NAC (10 mmol/L) or z-VAD-fmk (20 μmol/L) for 24 hours, and caspase-3 activation was determined by colorimetric assay. OD, optical density. C, PARP cleavage was detected by Western blotting.

Figure 3. Curcumin causes caspase-9 and -3 activation and PARP cleavage. A, HuT-78 cells were treated with different concentrations of curcumin (0–25 μmol/L) for 24 hours, and caspase-9 activity was determined by cell-based luminescence assay. Data represent mean values ± SD of 3 similar experiments. *P < 0.05 vs. control. B, cells were treated with curcumin (0–25 μmol/L) in the presence or absence of NAC (10 mmol/L) or z-VAD-fmk (20 μmol/L) for 24 hours, and caspase-3 activation was determined by colorimetric assay. OD, optical density. C, PARP cleavage was detected by Western blotting.

It is shown that Hsp90 promotes survival of cancer cells providing stability to pro- and antiapoptotic proteins (22). We, therefore, studied the effect of curcumin on Hsp90 expression. For this, HuT-78 cells were treated with different concentrations (0–25 μmol/L) of curcumin for 24 hours, whole-cell lysates were prepared and used for Western blotting. Significant cleavage of Hsp90 was observed when HuT-78 cells were treated with 25 μmol/L curcumin. This curcumin-mediated cleavage of Hsp90 was blocked by antioxidant, NAC (Fig. 4F). To see whether curcumin-generated ROS specifically cleaves Hsp90, we also checked the expression of Hsp70 in curcumin-treated HuT-78 cells. As shown in Fig. 4F, it is clear that curcumin has no effect on Hsp70 expression. Time kinetics experiment showed that Hsp90 cleavage started at 24-hour time point with 25 μmol/L curcumin (Fig. 4G). Hsp90 cleavage was inhibited by the addition of NAC or broad-range caspase inhibitor, z-VAD-fmk, with curcumin (Fig. 4H).

**Targeted disruption of Hsp90 by curcumin-induced oxidative stress, inhibited IKK-α/β, NF-κB**

It has been reported that disruption of Hsp90 leads to proteolytic cleavage of its client proteins (22). Thus, we checked the stability of Hsp90 client proteins IKK-α and IKK-β in curcumin-treated HuT-78 cells. Immunoblot analysis clearly showed that curcumin downregulated the expression of IKK-α and IKK-β in ROS-dependent manner as pretreatment of cells with NAC abolished the effect of curcumin (Fig. 5A). Interestingly, dose-dependent effect of curcumin was seen on IKK-α expression, whereas IKK-β expression was inhibited only at higher concentration of curcumin (25 μmol/L).

It is reported that curcumin inhibits constitutive NF-κB in CTCL cells (15). We speculated that inhibition of NF-κB activity may be due to IKK degradation in curcumin-treated cells. To see the status of NF-κB, nuclear extracts were prepared from various concentrations of curcumin-treated HuT-78 cells and subjected to EMSA. As shown in Fig. 5B, curcumin dose dependently inhibited NF-κB activity, and significant inhibition was observed when 25 μmol/L of curcumin was added. Curcumin-mediated inhibition of constitutive NF-κB was found to be under the control of ROS, as NAC pretreatment suppressed the inhibitory effect of curcumin. The specificity of the binding was examined by competition with unlabeled oligonucleotide (data not shown).

Next, we were interested to see whether Hsp90 is directly involved in NF-κB downregulation. For this,
HuT-78 cells were treated with different concentrations of Hsp90 inhibitor, 17-AAG and nuclear extracts were prepared and analyzed for DNA-binding activity of NF-κB by EMSA. Interestingly, at 2.5 μmol/L concentration, 17-AAG inhibits NF-κB DNA-binding activity (Fig. 5C).

**Hsp90 cleavage in curcumin-treated HuT-78 cells disrupts the stability of beclin-1**

Recently, it has been shown that Hsp90 forms a complex with beclin-1, a key protein involved in autophagy, and thus maintains the stability of beclin-1 (11). Because curcumin selectively cleaves Hsp90, therefore, we were interested to see the stability of beclin-1 in curcumin-treated HuT-78 cells. For this purpose, HuT-78 cells were treated with 25 μmol/L curcumin for 24 hours either in the presence or absence of NAC and thereafter, whole-cell lysates were prepared, and immunoblotting was done using anti-beclin-1 antibody. We observed that curcumin treatment resulted in the degradation of beclin-1, which was inhibited by NAC (Fig. 6A). Time kinetics experiments clearly indicated that degradation of beclin-1 occurred not before 24 hours of curcumin treatment (Fig. 6B). Similar result was obtained when similar experiment was carried out in MyLa cells (Fig. 6C).

Beclin-1 plays pivotal role in autophagy formation (23). Downregulation of beclin-1 in curcumin-treated cells indicated that the autophagy formation may be inhibited in this cell. Conversion of autophagy-specific marker LC3-I to LC3-II is an indicator for autophagy formation. We, therefore, monitored LC3-I to LC3-II conversion in curcumin (0–25 μmol/L)-treated HuT-78 and in MyLa cells. Accumulation of LC3-I was observed when higher concentration of curcumin (12.5 and 25 μmol/L) was present, which was inhibited by NAC (Fig. 6D). Rapamycin, a known inducer of autophagy was taken as positive control to induce autophagy and to check the conversion of LC3-I to LC3-II in HuT-78 cells. As expected, rapamycin treatment induced conversion of LC3-I to LC3-II, and this
conversion was inhibited in the presence of autophagy inhibitors, 3-MA and wortmannin, Wm (Fig. 6E).

Next, we studied the expression of autophagy-related proteins Atg5 and Atg7. Curcumin treatment inhibited the expression of Atg7 but not Atg5. Curcumin failed to show its effect in the presence of NAC (Fig. 6F).

Discussion

Recent years have seen a growing trend in developing therapeutic agents from natural sources against various diseases. Curcumin, a yellow pigment from turmeric that shows a wide spectrum of biologic function, has proved to be a promising candidate as an anticancer agent. Curcumin induces cell death in different forms of human cancer cells. Accumulating experimental data indicate that curcumin exerts its cytotoxic effect either by acting as an antioxidant or as a pro-oxidant (12). In this study, we have investigated whether curcumin treatment generates oxidative stress in HuT-78 cells, and if so, then what are the important cell survival mechanisms affected by curcumin-generated oxidative stress?

We report here that curcumin through ROS-dependent mechanism perturbs multiple cell signaling molecules and eventually induces apoptosis in HuT-78 cells. This conclusion is based on several crucial observations. First, time-dependent accumulation of ROS was observed in curcumin-treated cells and secondly, ROS scavenger, NAC, extensively attenuated all curcumin-induced effects such as activation of caspases, downregulation of antiapoptotic gene expressions, cleavage of Hsp90, inhibition of DNA-binding activity of NF-κB, including apoptosis in HuT-78 cells.

We have found that HuT-78 cells are much more vulnerable to oxidative stress than normal peripheral blood mononuclear cell (PBMC; data not shown). Induction of substantial apoptosis by curcumin in CTCL cells but not in PBMC from healthy donors, as reported recently by Zhang and colleagues, seems to be due to the generation of excessive ROS in those cancer cells (15). Apoptosis is mediated by the activation of different effector caspases. According to their function and mode of activation, caspases are classified as initiator caspases that include caspase-2, -8, -9, and executioner caspases such as caspase-3, -6, and -7. Active caspases are generated in 2 distinct pathways, the intrinsic or mitochondrial death pathway and the extrinsic or receptor-mediated pathway (6, 24). We have found that
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Figure 6. Curcumin-mediated oxidative stress degrades beclin-1, accumulates LC3-I, and inhibits Atg7. A, HuT-78 cells were treated with 25 μmol/L curcumin in the presence or absence of NAC (10 mmol/L) for 24 hours, and cell lysates were used to detect beclin-1, LC3-I, and LC3-II by immunoblotting. B and C, HuT-78 or MyLa cells were exposed with curcumin (25 μmol/L) for different time points, and beclin-1 expression was checked by Western blotting. D, HuT-78 or MyLa cells were treated with curcumin (0–25 μmol/L) in the presence or absence of NAC (10 mmol/L) for 24 hours, and cell lysates were used to detect LC3 by Western blotting. E, HuT-78 cells were treated with different concentrations of rapamycin (25 and 40 ng/μL) and then cell lysates were prepared. Immunoblot analysis with antibodies against LC3 or actin was conducted. 3-MA (10 mmol/L) or Wm (200 ng/mL) was added 1 hour before the addition of rapamycin (40 ng/μL). The ratios of intensities of LC3-II and actin are indicated below each lane. F, cells were treated with curcumin (0–25 μmol/L) for 24 hours, and cell lysates were prepared and analyzed for Atg5 and Atg7 by immunoblot analysis.

Curcumin through ROS generation activated caspase-8, -2, and -9 as well as caspase-3.

Caspase-8 activation is a crucial step for the initiation of the extrinsic pathway; on the other hand, caspase-9 activation is essential for the execution of intrinsic pathway. The link between these 2 pathways is mediated by the truncated proapoptotic protein Bid (6). Caspase-2 like caspase-8 is also known to cleave Bid to t-Bid that alters MMP (25). We observed truncation of Bid, mitochondrial hyperpolarization, cytochrome c release, caspase-9 activation followed by caspase-3 activation, and ultimately cleavage of PARP in curcumin-treated HuT-78 cells. All these mechanisms are controlled by ROS, as prior treatment with NAC completely blocked this cascade of events. Caspase-mediated pathways could not account for the 80% to 90% apoptosis induced by curcumin in HuT-78, as the addition of a broad-range caspase inhibitor as well as caspase-specific inhibitors (caspase-8 and -3) only partially suppressed cell death induced by curcumin.

Many researches have shown that curcumin influences multiple signaling pathways to exert its antiapoptotic effect on various cell types (12). Because we found that the caspase cascade is minimally involved in curcumin-mediated killing of HuT-78 cells; therefore, next we investigated the effect of curcumin on other cell survival molecules to find an explanation for curcumin-mediated killing of HuT-78 cells. It is known that HuT-78 cells express high amount of antiapoptotic proteins such as c-FLIP, Bcl-xL, cIAP, and XIAP. Bcl-xL, a member of Bcl2 family protein, is known to promote cell survival and regulate MMP (26). We found that curcumin at high concentration reduces the expression of Bcl-xL, which probably accounts for the alteration of mitochondrial membrane polarization seen in curcumin-treated cells. XIAP is known to regulate intracellular ROS by upregulating the expression of antioxidative genes (27). Many cancer cells have been shown to produce high amount of XIAP (28). We observed the cleavage of XIAP in curcumin-treated HuT-78 cells, which probably accounts for the high level of ROS generation by curcumin in these cells.

Researches have shown that Hsp90 function is required for the activation of constitutive IKK, which then activates constitutive NF-κB (10). We found that curcumin treatment results in degradation of IKK and as a consequence, downregulation of NF-κB takes place in HuT-78 cells in a ROS-dependent manner. Earlier Zhang and colleagues reported that curcumin inhibits constitutive NF-κB in CTCL cells (15); but for the first time, we showed the...
involvement of curcumin-generated ROS in the down-regulation of constitutive NF-κB in HuT-78 cells.

Curcumin has been shown to induce autophagy in different cell types (29, 30). It is also known that the IKK complex contributes to the induction of autophagy (31). Moreover, like IKK, beclin-1, the key autophagy-promoting protein, has been identified recently as a client protein of Hsp90 (11). We found the degradation of beclin-1 when HuT-78 cells were treated with 25 μmol/L curcumin. Time kinetics show that noteworthy downregulation of beclin-1 occurs at 24-hour time point, at the same time, when Hsp90 function is inhibited by curcumin in a ROS-dependent mechanism. Next, we investigated autophagy formation in curcumin-treated HuT-78 cells by following the conversion of autophagy-specific marker LC3-I to LC3-II. Accumulation of LC3-I rather than its conversion to LC3-II was visible at 25 μmol/L concentration of curcumin. Downregulation of autophagy-specific protein Atg7 occurs in curcumin-treated HuT-78 cells. Taken together, our results indicated that curcumin by disrupting Hsp90 also disrupts important cellular pathway, autophagosome formation, in a ROS-dependent manner. Cell proliferation and cell survival are regulated by a complex interactive network of cell signaling pathways. Therefore, it may not be sufficient to control malignant cell growth simply by disrupting 1 or 2 cellular targets. Research has established that one common biochemical change in malignant cells is the increased production of ROS due to high metabolism (21). This fact is used to develop therapeutics against malignant cells. Curcumin successfully uses this strategy to induce oxidative stress in HuT-78 cells, perturb important cell survival mechanisms, and thus achieve high degree of killing.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

Authors’ Contributions

Conception and design: M.A. Khan, S. Gahlot
Development of methodology: M.A. Khan, S. Gahlot
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.A. Khan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.A. Khan
Writing, review, and/or revision of the manuscript: M.A. Khan, S. Gahlot
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.A. Khan
Study supervision: S. Majumdar

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