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Therapeutic potential of curcumin in human prostate cancer—I. curcumin induces apoptosis in both androgen-dependent and androgen-independent prostate cancer cells

T Dorai[1]*, N Gehani[1] & A Katz[1]

[1]*Department of Urology, Columbia University, College of Physicians and Surgeons, New York, NY 10032, USA*

In an effort to find an alternative nontoxic means of inducing the apoptosis potential in both androgen-dependent and hormone refractory prostate cancer cells, attention was focused on curcumin (turmeric), traditionally used in medicine and cuisine in India and other south-east Asian countries. The results indicate that curcumin is a novel and potent inducer of apoptosis in both androgen-dependent and androgen-independent prostate cancer cells. This was accomplished by down-regulating apoptosis suppressor proteins and other crucial proteins such as the androgen receptor. It is concluded that curcumin may provide an alternative, nontoxic modality by which the clinician may prevent the progression of prostate cancer to its hormone refractory state or to treat advanced prostate cancer by forcing them to undergo apoptosis. *Prostate Cancer and Prostatic Diseases* (2000) **3,** 84–93.

Keywords: prostate cancer; complimentary medicine; curcumin; turmeric

Introduction

Prostate cancer is the most common neoplasm and the second leading cause of male death in the USA.[1] The inability and the frustration in the clinical management of prostate cancer is derived from the fact that, despite the development of several androgen ablative therapies, an effective therapeutic regimen is still lacking, especially for the treatment of hormone refractory prostate cancer.[2,3] Therefore, development of mechanism-based treatment strategies is desperately needed. As a way to approach this problem, several investigators have recently turned towards strategies in alternative medicine, tapping into the vast traditional knowledge base available in various ethnic societies. Over the past few years, a number of foods, vitamins and minerals have been found to be beneficial for the prevention and treatment of prostate cancer and other cancers. Selenium, vitamins A, C and E, garlic, green tea, soy bean, PC-SPES, to name a few, have been the topic of discussion in many recent publications.[4–9] Efforts are being made in several laboratories, including ours, to augment, improve and supplement the current cure rates of prostate cancer by conventional therapies such as androgen ablation, radiation and chemotherapy with nature-based therapies. Evidence is mounting that we can prevent many illnesses such as prostate cancer by modifying our lifestyle, such as diet.[10]

In an effort to search for alternative therapies for hormone refractory prostate cancer, attempts are being made in this laboratory to determine the efficacy of some holistic remedies and study the molecular mechanisms by which some dietary ingredients such as curcumin, traditionally used in India and other south-east Asian countries for thousands of years, influence the genesis and progression of human prostate cancer to its advanced stages. Curcumin, popularly called turmeric (diferuloyl methane), the powdered rhizome of *Curcuma longa* L, has been used traditionally as a coloring and flavoring additive in Indian and south-east Asian cuisine. It was also used to treat a variety of inflammatory conditions and chronic diseases.[11–16] It displays anti-carcinogenic properties in animals, as indicated by its ability to inhibit both

*Correspondence: T Dorai, Comprehensive Cancer Center, Our Lady of Mercy Medical Center, New York Medical College, 600 East 233rd Street, Bronx, NY 10466-2697, USA.
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tumor initiation induced by benzo(a)pyrene-mediated DNA adduct formation and development of skin tumors, as well as tumor promotion induced by phorbol esters, possibly by inhibiting protein kinase C.[17] Importantly, dietary administration of curcumin during the initiation and post-initiation periods significantly suppressed the development of chemically induced forestomach, duodenal and colon tumors in CF-1 mice.[18] It also reduced the formation of focal areas of dysplasia and aberrant cyptic foci in the colon that are the early preneoplastic lesion in the rodents.[19] In another important study, Kawamori *et al* showed that chemopreventive activity of curcumin could be observed even in the promotion/progression phase of colon carcinogenesis in experimental animals.[20] Consistent with the chemopreventive activity during all states of tumor formation, curcumin has also been shown to decrease the expression of proto-oncogenes such as c-*fos*, c-*jun* and c-*myc*, possibly through the inhibition of specific protein kinases.[21] Curcumin also has anti-tyrosine kinase activity against EGF-receptor in A431 cells and $p185^{neu}$ in AU-565 cells.[22,23] Moreover, several recent studies reported the occurrence of apoptosis or apoptosis like changes upon exposure to curcumin in human lymphoblastoid T (jurkat) cells and in human promyelocytic leukemia (HL-60) cells.[24,25] Because curcumin has already been shown to have negligible systemic toxicity in animal and human studies and since it has been classified under the 'generally recognized as safe' or 'GRAS' category by the chemoprevention branch of the National Cancer Institute (NCI), and since no study has been reported on the efficacy of curcumin in interfering with prostate carcinogenesis, we explored the effect of curcumin on cell proliferation and its apoptosis-inducing potential in human prostate cancer cells.[26–28]

Materials and methods

Cell culture

The androgen-sensitive human prostatic carcinoma cell line LNCaP and its bcl-2 over-expressing relative (LNCaP-bcl-2) were maintained as described previously.[29] The androgen-independent prostate cancer cell line PC-3 was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained as recommended. All cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Treatment with curcumin and cell viability assays

The various prostate cancer cell lines were seeded in 96-well plates at a density of 5×10^3 cells in 100 µl per well. Curcumin was purchased from Sigma and stored as a 50 mM stock solution in DMSO, protected from light at 20°C. Twenty-four hours after seeding, the medium was removed and replaced with fresh medium containing DMSO (vehicle only) or medium containing increasing concentrations of curcumin (usually 5–50 µM) in a total volume of 100 µl, and the culture was maintained in the CO_2 incubator for a further period of 3 days. For each curcumin concentration and each time point, cultures were maintained in quadruplicates. At the end of the 24, 48 and 72 h periods, 10 µl of the MTT labeling solution was added from a cell proliferation assay kit obtained from a commercial source (Roche Diagnostics, Indianapolis, IN) and processed exactly as described by the manufacturer. After the solubilization of the purple formazan crystals was complete, the absorbance of each well was measured spectrophotometrically using a microtiter plate reader, using a filter for 570 nm.

Western blot analysis

(a) Apoptosis modulating proteins LNCaP parental cell and LNCaP-bcl-2 cells were treated with increasing concentrations of curcumin. The relative levels of apoptosis suppressor proteins such as bcl-2, bcl-X_L and apoptosis promoter proteins such as bax were measured against a backdrop of actin protein, which was used as a negative control, using methods described previously.[29] The cells were lysed by treatment in RIPA buffer (10 mM Tris–HCl, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl containing 50 µg/ml of antipain, 40 µg/ml of pepstatin, 30 µg/ml of chymostatin, 1 µg/ml of leupeptin, 1 µg/ml of pepstatin, µg/ml of aprotinin and 1 mM phenyl methyl sufonyl fluoride. Protein concentration was measured using a modified Bradford assay (Bio Rad Laboratories, Hercules, CA). A blocking solution of 5% BSA was used instead of non-fat milk. The antibodies used were a mouse monoclonal anti-bcl-2 from Dako (Dako Corp, Carpinteria, CA), at 1 : 3000 dilution; a mouse monoclonal anti bcl-X_L, from Santa Cruz (Santa Cruz, CA), at 1 : 3000 dilution; and a mouse monoclonal antibody for bax (Santa Cruz, CA, at 1 : 3000 dilution). After washing in Tris buffered saline containing 0.05% Tween 20, the filters were incubated with a 1 : 5000 diluted and corresponding secondary antibody conjugated with horseradish peroxidase (Santa Cruz, CA). After washing, the filters were treated with the enhanced chemiluminescence (ECL) solution mixture exactly as described by the manufacturer (Amersham, Arlington Heights, IL). The filters were exposed to X-OMAT AR X-Ray film for times ranging from 30 s to 5 min.

(b) Markers relevant to prostate cancer Normalized amounts (25 or 50 µg) of proteins from the RIPA extracts prepared as described earlier were processed for Western blots in either 7.5% or 12% gels depending upon the size of the protein in question. The blots were blocked, probed and processed for ECL as described earlier with a monoclonal antibody for the human androgen receptor (Sata Cruz, sc-7305, at 1 : 3000 dilution) or with a mouse monoclonal antibody for the human prostate specific antigen (Santa Cruz, sc-7316 at 1 : 3000 dilution).

Analysis of apoptosis induced by curcumin

(a) Detection of apoptotic nuclei by the TUNEL method For this analysis, parental LNCaP cells were grown on cover slips as described above. Enzymatic *in situ* labeling of apoptosis-induced DNA strand breaks were done by the TUNEL (TdT mediated dUTP nick end labeling) method,

using a commercially available kit (Roche Diagnostics, Indianapolis, IN), following a protocol described by the manufacturer.

(b) Detection of apoptotic cells by Annexin-V staining As an early indicator of apoptosis in LNCaP prostate cancer cells in response to exposure to curcumin, the rapid translocation and accumulation of the membrane phospholipid phosphatidyl serine (PS) from the cytoplasmic surface to the extracellular surface was followed. This loss of membrane assymetry was detected by using a FITC-conjugated PS-binding protein Annexin-V (Santa Cruz). LNCaP cells, grown in cover slips as described earlier, were treated with the drugs for 12 h (instead of 24 h as described for DAPI or TUNEL staining procedures), and were washed free of the medium and incubated with the assay buffer containing Tris buffered saline with 2.5 mM $CaCl_2$ for 10 min at room temperature. The cover slips were then treated with Annexin-V-FITC diluted in the same assay buffer exactly according to the manufacturer's protocol. The cover slips were then mounted on glass slides and visualized using an Olympus fluorescence microscope with a filter set for FITC.

(c) Analysis of apoptosis by PARP cleavage Parental LNCaP cells were exposed to increasing concentrations of curcumin as described earlier for 24 h. The floating and adherent cells were pooled, washed three times with 1×PBS to remove the medium and lysed in 1×RIPA buffer (10 mM Tris HCl,pH 7.4, 1% NP-40 0.5% sodium deoxy cholate, 0.1% SDS, 0.15 M NaCl containing a protease inhibitor cocktail as described earlier, (Roche Diagnostics, Indianapolis, IN). Normalized amounts of cell lysate protein (25 μg) were electrophoresced through 7.5% denaturing polyacrylamide gels. The resolved proteins were transferred to nitrocellulose and probed with a mouse monoclonal antibody against the human Poly ADP-Ribose Polymerase (PARP, Santa Cruz Biotechnology, Santa Cruz, CA) as described earlier in the Western blotting protocol and the ECL procedure was performed to visualize the protein bands.

Results

Curcumin diminishes the proliferative potential of human prostate cancer cells

As practically no published information was available on the effect of curcumin on the prostate system, our initial experiments focused on the effect of curcumin on the cell growth and proliferative potential of prostate cancer cells. LNCaP was chosen as the androgen-dependent prostate cancer cell line and PC-3 was chosen as the androgen-independent cell line, and they were grown in 96-well plates. Curcumin, dissolved in DMSO, was used at a concentration range between 0 and 50 μm. The cancer cell lines were exposed to the drug one day after seeding and their viability was followed for 3 days using the MTT cell proliferation assay. Results are presented as percentage of control growth on the corresponding day and each value was measured in quadruplicate. The cell proliferative potential of LNCaP and PC-3 are shown in Figure 1A and B, respectively. The figures shown that exposure to curcumin at a range between 20 and 50 μM strongly inhibited the growth of both cell lines by 60–80%, indicating that this drug affects the cell proliferative rate of both androgen-dependent and androgen-independent prostate cancer cells in a dose-and time-dependent manner. The kinetics of the MTT assay curves suggest that the effect of curcumin could be both cytostatic and cytocidal. There was no growth inhibition of any of these prostate cancer cell lines by the DMSO vehicle (used at the same final concentration of 0.1%). Later, another androgen-independent prostate cancer cell line DU-145 was also shown to be inhibited by exposure to curcumin in a similar manner (data not shown). There was a marked change in the morphology within 48 h of curcumin treatment, characterized by more round and floating cells, gradually increasing from concentrations from 20 to 50 μM. Results of other experiments showed that there was no effect of curcumin on the general protein synthesis of parental LNCaP cells by exposure to 20 μM curcumin for 6 h and the growth inhibition of LNCaP cells by curcumin (30 μM for 24 h) was not reversible (data not shown). It is worth noting that the IC_{50} of curcumin on both LNCaP and PC-3 cells ranges from 10 to 20 μM, which is close to that of some of the conventional chemotherapeutic agents such as 5-fluorouracil.[30] Moreover, since animal and human studies have shown that curcumin is generally safe with no untoward systemic toxicity, the potential therapeutic role of curcumin in androgen-dependent and hormone refractory prostate cancers merits further study, in addition to its well-established role as a chemopreventive agent.

Over-expression of bcl-2 does not protect LNCaP cells from the effects of curcumin:

The LNCaP derivative over-expressing the apoptosis suppressor protein bcl-2 has already been described in the earlier work from this laboratory.[29] Our previous work has shown that, while not affecting the proliferative properties or the expression of differentiated prostate specific gene products such as PSA and the androgen receptor, bcl-2 expression in LNCaP cells conferred resistance to apoptosis when these cells were grown in an androgen/steroid depleted medium *in vitro* and *in vivo* (ie after castration).[29] The derivatized cells were also resistant to several other apoptosis stimuli such as serum starvation and phorbol ester treatment. These earlier data suggested that the bcl-2 oncoprotein can protect prostate cancer cells from apoptosis *in vitro* and such protection correlates with the tumor-forming ability in the absence of androgens *in vivo* and could from a central working model for the genesis and maintenance of hormone refractory prostate cancer.[31] So, attempts were made to explore the role of bcl-2 protein in curcumin-induced decrease in cell proliferation. Interestingly, the results shown in Figure 1C indicated that curcumin can decrease the proliferative potential of bcl-2 over-expressing LNCaP cells too in a time-and concentration-dependent manner, in the same way as the parental LNCaP and PC-3 cells. As a negative control, LNCaP cells expressing only the expression vector (neo®) were used and were

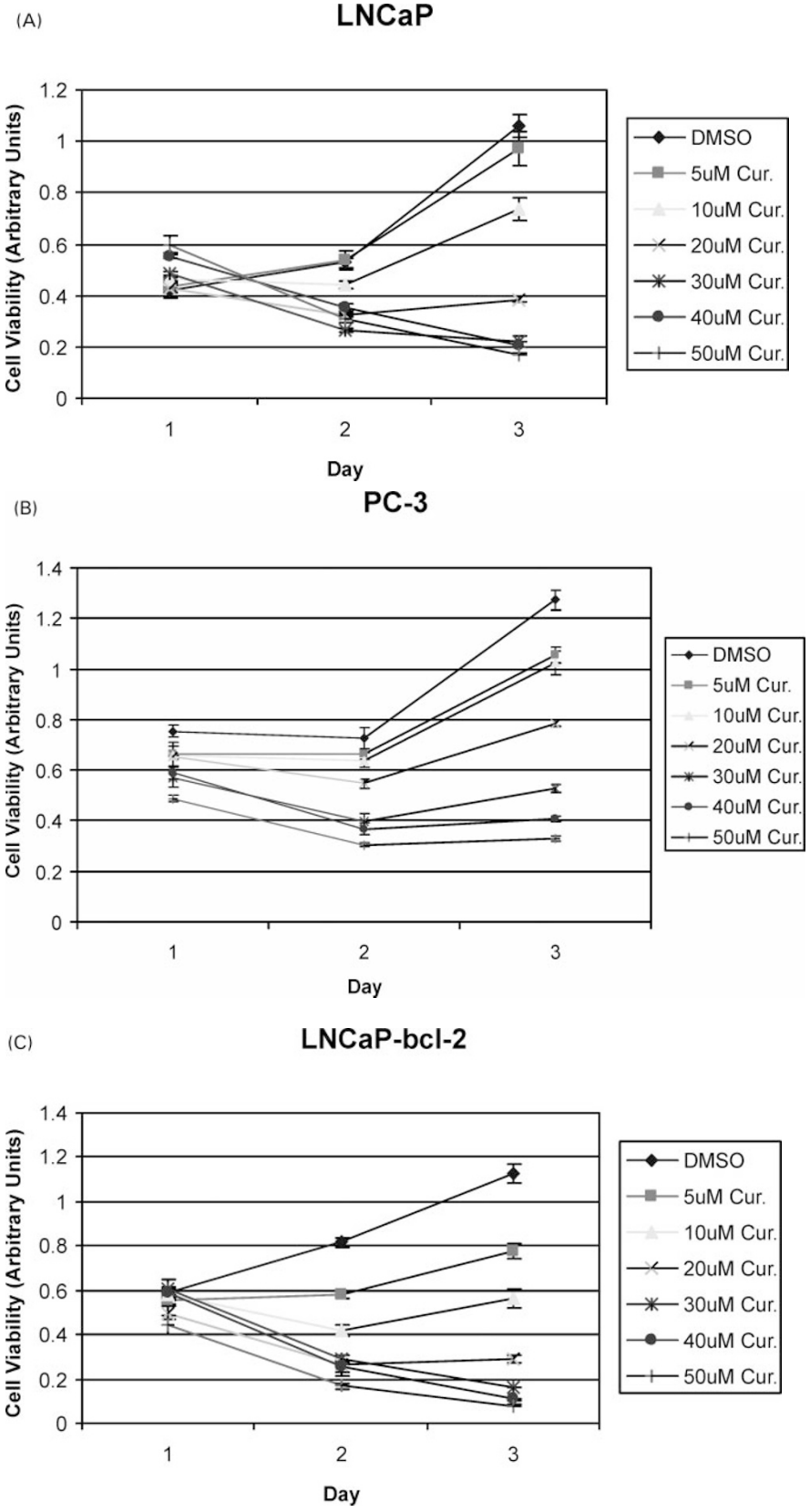

Figure 1 *Treatment of parental androgen-dependent LNCaP cells (A), androgen-independent PC-3 cell (B) and LNCaP cells over expressing bcl-2 proto-oncogene (C) with curcumin promotes loss of cell viability. Twenty-four hours after seeding (day 0), cultures were supplemented with different doses of curcumin, as indicated. Cell growth was measured by the MTT cell proliferation assay. Each time point was measured in quadruplicates using 96-well culture plates and expressed as relative cell viability with respect to the untreated control for that corresponding day.*

found to behave exactly the same way as the parental LNCaP (data not shown). Later, curcumin was also found to inhibit the proliferative potential of LNCaP cells over-expressing another apoptosis suppressor protein, bcl-X_L (data not shown). These results strongly suggest that the expression of apoptosis suppressor proteins fail to protect prostate cancer cells from cell death induced by curcumin. This would imply that curcumin may be able to suppress cell proliferation and induce cell death through bcl-2 or bcl-X_L-independent mechanisms.

Curcumin down-regulates the levels of apoptosis suppressor proteins and modulates the bax/bcl-2 ratio

Next, we analyzed the changes in the expression of apoptosis modulating proteins such as bcl-2, bcl-X_L and bax in parental LNCaP cells and compared them with the level of expression of a housekeeping gene such as actin at the protein level, in response to the exposure to curcumin at different concentrations (0–50 μM). As shown in Figure 2A, the bcl-2 and bcl-X_L levels are strikingly down-regulated by incubation with curcumin above a concentration of 20 μM in a period of 24 h. Parental LNCaP cells are known to express trace amounts of bcl-2 protein, which could be observed only after long exposure of the ECL blots to the X-ray films.[29] In contrast, proteins such as bcl-X_L and bax are expressed at a higher level than bcl-2 in LNCaP cells.[32] The expression of bcl-2 protein is undetectable at 30 μM concentration of curcumin or higher after a 24 h exposure. Similar decrease was observed with respect to the level of expression of another apoptosis suppressor protein bcl-X_L. Interestingly, one other protein crucial to the regulation of the apoptotic process, namely bax, was essentially unchanged after exposure to curcumin. As a negative control, identical amounts of protein extracts were electrophoresced and the blots were probed for actin. This analysis showed negligible change after exposure to increasing concentrations of curcumin. Next, we examined whether the decrease in apoptosis suppressor protein bcl-2 would be true for bcl-2 over-expressing cell also. The results of the immunoblots of bcl-2 over-expressing LNCaP cells are shown in Figure 2B. A dramatic down-regulation of bcl-2 levels was seen in bcl-2 over-expressing cell too, as seen in the figure. In contrast, LNCaP cells transfected with the neo-resistant expression vector only (LNCaP-neo®) behaved exactly like parental LNCaP cells (data not shown). These results highlight the intriguing property of curcumin in selectively down-regulating the apoptosis suppressor proteins while the levels of apoptosis promoting proteins are relatively unaffected. This would theoretically lead to a scenario where the bax/bcl-2 or bcl-X_L ratio would be increased, pushing the cells towards initiating and progressing through the apoptotic process.

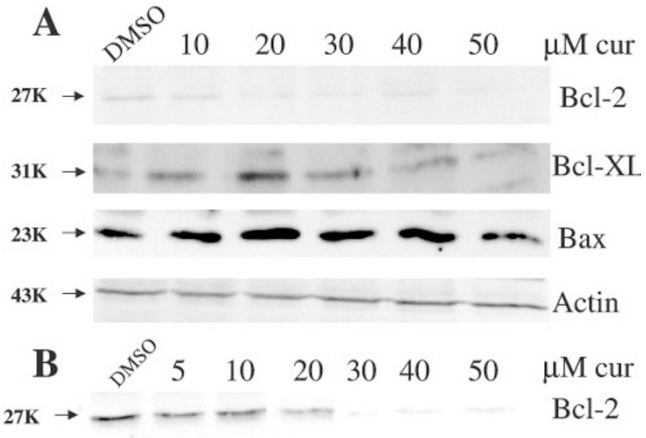

Figure 2 *Curcumin treatment modulates the levels of apoptosis suppressor proteins bcl-2 and bcl-X_L, while that of the pro-apoptotic protein bax is relatively unaffected. (A) Western blot analysis done on normalized amounts (25 μg each) of protein extracts prepared from the parental LNCaP cells. (B) Similar protein extract prepared from LNCaP cells over-expressing the bcl-2 protein. As controls, the Western blots were probed with antibodies against bax and actin, as shown (A).*

Curcumin induces apoptosis in both androgen-dependent and androgen-independent prostate cancer cells in a concentration-dependent manner

To determine whether curcumin-induced cell death demonstrates the characteristic features of apoptosis, we examined parental untreated LNCaP cells, LNCaP cells treated with 30 μM curcumin for 24 h or LNCaP cells treated for 24 h with 10 nM phorbol myristate acetate (PMA) as a positive control using several methods commonly used for the analysis of the apoptotic process.[29] The apoptotic DNA ladder pattern of curcumin-treated prostate cancer cells on agarose gels was not attempted, as this method provides neither information regarding apoptosis in individual cells nor information regarding histological localization. Hence, the enzymatic *in situ* labeling of apoptosis-induced DNA strand breaks using terminal deoxynucleotidyl transferase (TUNEL) procedure was performed on all three cell systems described above. The final signal was converted with an anti-fluorescein antibody conjugated with horseradish peroxidase and visualized under a light microscope. As can be seen in Figure 3A, intense staining of the curcumin-treated nuclei could be observed, which was qualitatively comparable with the TUNEL stain that could be obtained after treating parental LNCaP cells with phorbol myristate acetate as a positive control. In order to further characterize the morphological features to curcumin-induced cell death, one of its earliest features, namely the changes in the plasma membrane, especially the translocation of the membrane phospholipid phosphatidyl serine (PS) from the inner leaflet to the outer leaflet of the plasma membrane was followed by the binding of the fluorescein isothiocyanate (FITC)-conjugated PS binding protein Annexin-V in the presence of Ca^{2+} ions. Loss of membrane integrity and externalization of PS could be observed in parental LNCaP treated with either curcumin or the PMA positive control, as early as 12 h after drug treatment, as seen by the intense FITC fluorescence in Figure 3B as compared with the untreated control. Finally, in order to verify whether poly ADP ribose polymerase (PARP) proteolysis could be observed as an indicator of activation of the PARP protease (CPP32/prICE) as an early response to treatment of parental LNCaP cells with curcumin, Western blot analysis was performed on LNCaP cell lysates, treated with increasing concentrations

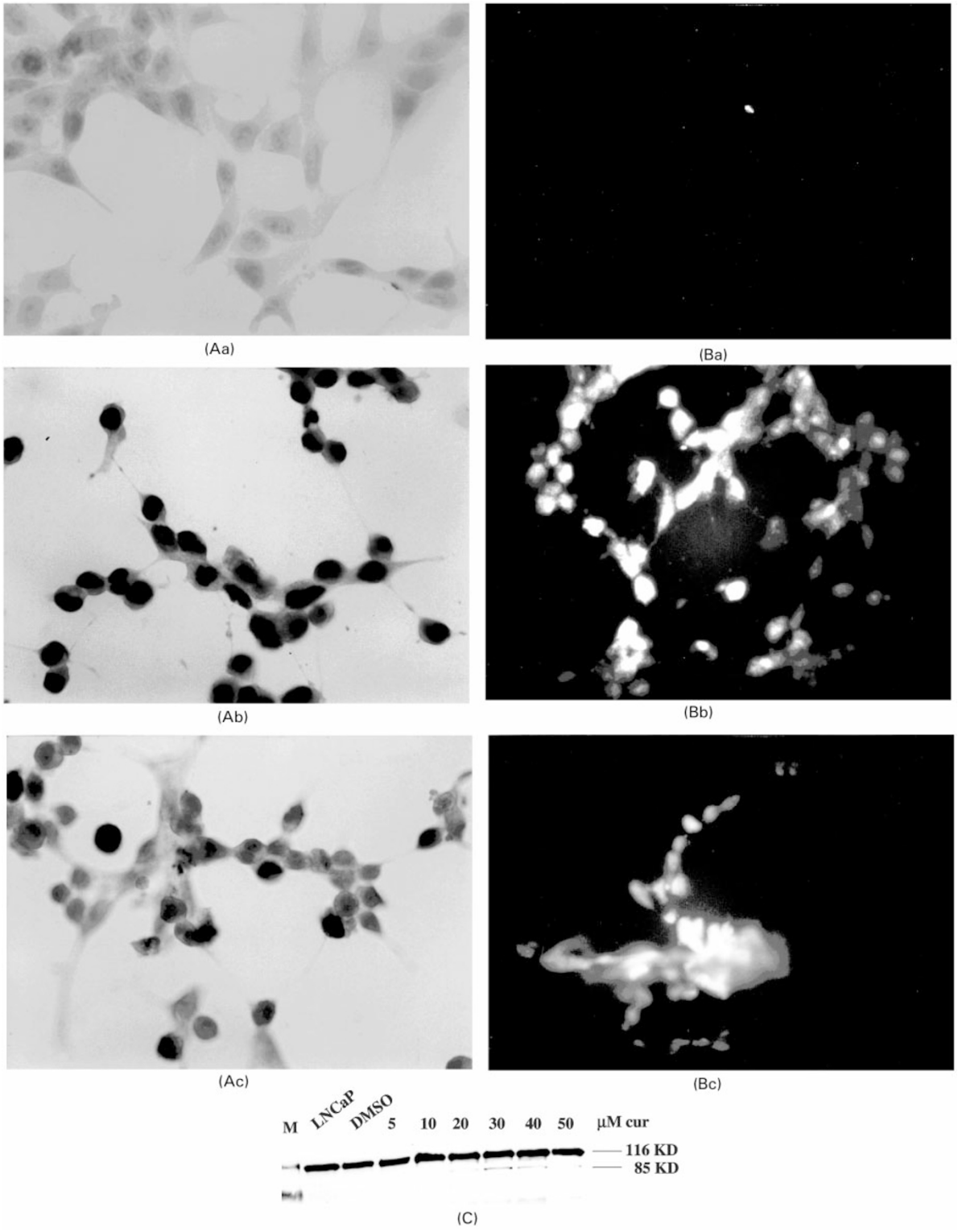

Figure 3 *Apoptosis in parental LNCaP cells as visualized by several methods after curcumin (test) or phorbol myristate acetate (PMA, positive control) treatment. (A) TUNEL, staining of parental LNCaP cells treated with drugs as described in (A). The TUNEL positive cells are visualized by their dark brown nuclear staining. The cells, prepared as described in (A), were processed for the TUNEL staining method exactly as described by the manufacturer and finally counter-stained by hematoxylin before analysis by light microscopy (magnification 20×). (a) No treatment; (b) 30 μM curcumin treatment; and (c) 10 nM PMA treatment. (B) Annexin-V-FITC staining of parental LNCaP cells treated with drugs as described in (A) except that the time of treatment was 12 h. Annexin-V-FITC binding was done in the presence of Ca^{2+} ions. (a) No treatment; (b) 30 μM curcumin treatment, and (c) 10 nM PMA treatment. The fluorescence is seen in black and white due to a digital transfer of the computer images from the fluorescence microscope. Please see Methods for experimental details. (C) Further analysis of the apoptotic process in LNCaP cells after exposure to curcumin. Cleavage of poly ADP-ribose polymerase (PARP) protein in parental LNCaP cell after treatment with increasing concentrations of curcumin. The 85 KDa cleavage product of native PARP (116 kDa) which is recognized by the antibody is indicated.*

of curcumin. The results are shown in Figure 3C. Proteolysis of PARP from the native 116 kDa protein to a characteristics 85 kDa fragment was visible from a curcumin concentration of 20 μM and above. All these data strongly suggest that curcumin treatment of the classical prostate cancer cell line LNCaP in accompanied by the phenomenon of programmed cell death.

Curcumin-induced apoptosis of LNCaP cells is accompanied by a down-regulation of the androgen receptor (AR) protein more dramatically than prostate-specific antigen (PSA)

Next, we wished to determine whether curcumin treatment of parental LNCaP cells, while initiating the process of apoptosis, had an influence on the level of expression of typical prostate marker proteins such as the androgen receptor protein and prostate-specific antigen. The results of the Western blot analysis are shown in Figure 4. As can be seen from Figure 4A, the androgen receptor protein is strikingly down-regulated in response to curcumin treatment. The reduction in the protein levels could be seen at curcumin concentrations as low as 10 μM and the AR, protein is virtually nonexistent at 30 μM. Interestingly, a Western blot analysis performed on the same cell lysates revealed a less dramatic reduction in the level of expression of the prostate-specific antigen protein, shown in Figure 4B. There was no change in the level of expression of the actin protein, which was found to be essentially the same as shown in Figure 2A (data not shown).

Discussion

This study was undertaken as part of an exploratory screening of holistic phytochemicals that are well known in specific ethnic societies and have both preventive and therapeutic potential in cancer, and to translate their potential into a clinical prostate cancer setting. One such candidate under consideration, namely curcumin, has received a lot of encouragement in recent studies, which reported that it could not only interfere with the initiation/promotion period of colon carcinogenesis but also with the promotion/progression period, meaning that it could be potentially useful in the treatment of pre-existing neoplasms.[17–20] Although curcumin has

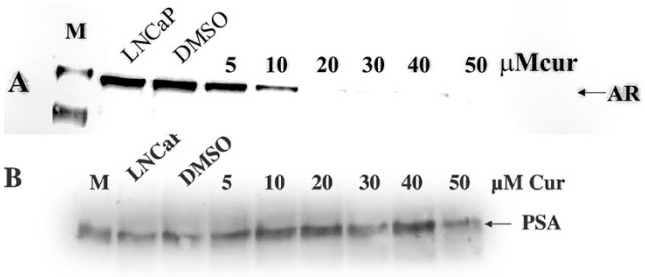

Figure 4 *Modulation of the androgen receptor (AR) levels (A) and prostate-specific antigen (PSA) levels (B) in parental LNCaP cells after treatment with increasing concentrations of curcumin as analyzed by Western blots. The specific AR and PSA bands are indicated by arrows. Please see Methods for other experimental details. Normalized amounts of proteins in RIPA extracts were used for the protein analysis (25 μg for AR protein and 50 g for PSA antigen).*

several metabolic, cellular and molecular activities, recent studies have suggested that it can also act through mechanisms such as inhibition of cell proliferation and induction of apoptosis in colon tumors.[33] Hence, a hypothesis was proposed in this study that curcumin, on the basis of its established properties, may inhibit the proliferative potential and induce the apoptotic potential of prostate cancer cells and affect the signal transduction pathways so as to interfere with the progression of prostate cancer to its hormone refractory state.

Androgens regulate the growth and development of the prostate gland and are necessary for the maintenance of its functions. As the mainstay of the therapeutic modality for prostate cancer, androgen ablation methods induce an initial and impressive wave of apoptosis, by which most of the androgen-withdrawal sensitive prostate cancer cells are eliminated. However, a certain population of prostate cancer cells, called the androgen-withdrawal insensitive prostate cancer cells, which either by virtue of their pre-existence or by adaptation in an androgen depleted mileau, learn how to survive.[34] This is largely due to an enhanced expression of apoptosis suppressor proteins such as bcl-2 and bcl-X_L in prostate cancer cells, which subsequently gain growth advantage to proliferate and metastasize to distal organs with a fatal outcome.[35,36] Such a possibility was suggested by earlier work from our laboratory and others that bcl-2 over-expression in LNCaP cells confers an androgen-independent phenotype in LNCaP cells which enables them to grow in an androgen depleted medium *in vitro* and grow *in vivo* in castrated nude mice.[29,37] Also, it is worth noting that an enforced expression of bcl-2 causes a more invasive phenotype in LNCaP cells.[38,39] Hence, there is a desperate need to discover a non-androgen ablative means to kill the androgen-insensitive cancer cells of the prostate which do over-express these apoptosis suppressor proteins. In this regard, several important publications have revealed the fact that, despite offering resistance to several apoptotic stimuli such as androgen withdrawal, serum starvation or phorbol ester treatment, androgen-insensitive prostate cancer cells do in fact maintain the basic and apparently functional apoptotic machinery which could be activated under certain circumstances.[40,41] So, a central aim of this study has been to test the hypothesis that the apoptotic machinery of the prostate cancer cells may be activated despite the presence of apoptosis suppressor proteins such as bcl-2 and bcl-X_L for tumoricidal purposes and that the unwanted hormone refractory prostate cancer cells can be forced to commit to apoptosis. Hence, a down-regulation of proteins such a bcl-2 and bcl-X_L will have significant therapeutic benefits in a major fraction of prostate cancer patients who over-express these proteins. In this respect, curcumin offers itself as the most promising candidate in that it induces apoptotic potential not only in androgen-sensitive prostate cancer cells such as LNCaP, but also in PC-3 cells and in the androgen-insensitive LNCaP cells with an enforced expression of the apoptosis suppressor proteins bcl-2 or bcl-X_L.

Earlier work from this laboratory also suggested that ablation of bcl-2 expression with the help of a specific anti-bcl-2 ribozyme promoted apoptosis in bcl-2 expressing human prostate cancer cells.[42–44] But the results reported in this study reveal the facts that first, curcumin

may even be superior to agents like the mRNA-specific ribozyme in that it down-regulates not only bcl-2 but also bcl-X_L; second, no gene therapeutic adenoviral or retroviral protocols need to be drawn; third, it can be used both in the chemopreventive and the therapeutic modes by introduction in the diet; and fourth and most importantly, curcumin exerts its beneficial effects at a negligible fraction of the cost compared with the gene therapy protocols or even the natural estrogen-based protocols such as PC-SPES.[45] The most important statement that one can make from this study is that curcumin is probably the most effective and the least expensive of all reagents by which one can bypass the apoptosis resistance of hormone refractory prostate cancer cells. It would be very interesting to see whether curcumin can still activate proteases such as caspases 3 and 7 and facilitate the accumulation of cytochrome c in the cytosol despite an over-expression of bcl-2 in prostate cancer cells.[46] Because caspase activation occurs downstream of the bcl-2 checkpoint, the effect of curcumin may have significant clinical implications.[47] It is highly possible that curcumin activates specific caspases that induce apoptosis in prostate cancer cells that over-express bcl-2 and bcl-X_L. Studies are under way in the laboratory to decipher these processes. Also, it should be mentioned here that, while the effect of curcumin meets most of the stringent criteria set for the phenomenon of apoptosis such as the modulation of bcl-2 and bcl-X_L levels, DAPI and TUNEL staining and Annexin-V staining, the cleavage of the PARP protein precursor to the 85 kDa proteolytic fragment is not quantitative, unlike other apoptosis inducers such as etoposide, which raises the possibility that curcumin may bring up other forms of cell death in addition to the apoptotic cell death process.[25]

Bax and bcl-2 are two discrete members of the gene family involved in the regulation of cellular apoptosis. Even though these two genes share partial amino acid sequence homology in their functional domains, their individual protein products appear to have opposing functions with regards to their effects on the apoptotic sensitivity of a cell. Pro-apoptotic bax and anti-apoptotic bcl-2 family members can heterodimerize and seemingly titrate out each other's function, suggesting that their relative concentration may act as a rheostat for the cell suicide program in the sense that their intracellular ratio can profoundly influence the ability of a cell to respond to an apoptotic signal. Even though the current experimental evidence indicates that there are a few subtle exceptions , as a general rule of thumb, the rheostat theory still holds good in that a cell with a higher bax/bcl-2 ratio will be more sensitive to a given apoptotic stimulus when compared to a similar cell type with a relatively low bax/bcl-2 ratio.[48,49] The modulation of protein levels in bax and bcl-2 shown in this study is consistent with and supports this view.

The observation that curcumin is able to down-regulate bcl-X_L proto-oncogene too, apart from bcl-2 in the prostate cancer cells, would have more theoretical and therapeutic implications. Bcl-X_L protein expression has also been found in the epithelial cells of the normal prostate gland, where it has been speculated to contribute to the hormonal control of programmed cell death.[36] In the same publication, it was reported that out of 64 cases of the adenocarcinoma of the prostate, all (100%) stained positively for the bcl-X_L protein. More over, the intensity of staining and the percentage of immunopositive cells seemed to be directly correlated with the Gleason score.[36] Whereas bcl-2 over-expression is found in a small subset of the adenocarcinomas of the prostate, it is found far less commonly than bcl-X_L.[35] When over-expressed in cell systems such as the prolymphocytic leukemia cell line FL5.12, bcl-X_L, was found to profoundly interfere with the apoptotic stimuli of various chemotherapeutic agents.[50] It has been proposed that the presence of the anti-apoptotic protein bcl-X_L expressed in prostate cancer cells may be related to the relative resistance to chemotherapy in clinical prostate cancer. This observation, when added to the differences in the subcellular localization between these two proteins, would strongly suggest that these two proteins are under two different regulations and that bcl-2 and bcl-X_L would modulate different paths in the prevention of apoptosis in the prostate cancer cells that are mutually exclusive or independent.[51,52] It is significant that, in spite of these differences, curcumin is able to bypass the two different styles of apoptosis resistance mediated by these two key players, by interfering with a common denominator in their function which is yet to be characterized.

Androgen receptor is a critical factor for cell growth both in the normal prostate and in prostatic carcinoma. Prostate tumors, especially the ones as exemplified by LNCaP, adapt to an environment of low or no androgen supply by using a hyperactive androgen receptor. In approximately 30% of cases in which the tumors re-emerge after androgen ablation, cancer cells show high levels of AR gene amplification with a concomitant elevation in the level of functional AR.[53] Irrespective of the AR gene amplification, increased AR expression appears to be the single and selective driving force for the progression of prostate cancer to the hormone refractory phase.[54] A similar phenomenon was observed in the LNCaP prostate cancer derived subline 104-R.[55] In this report, transition from the clonal subline LNCaP 104-S during prolonged passage in an androgen-depleted medium to the subline 104-R was accompanied by an accelerated growth phase and a 15-fold increase in the androgen receptor protein level compared with the parental LNCaP controls. Thus, prostate cancer that recurs after androgen deprivation therapy is clearly androgen receptor dependent if not androgen dependent. However, the exact mechanism that produces a high level of activated androgen receptor is still unknown and may be due to the presence of growth factors or protein kinase A mediated pathways that elicit a ligand-independent activation of the androgen receptor.[56,57] Irrespective of the activation mechanism, a specific down-regulation of the androgen receptor protein in LNCaP cells after curcumin treatment, as observed in this study, is expected to deprive these cells of a critical growth advantage and qualifies this phytochemical for a nontoxic therapeutic approach to the management of AR-dependent prostate cancer.

In conclusion, curcumin, whose medicinal properties are mentioned in ancient vedic scriptures of India, may be the most novel and appropriate naturally occurring reagent ever (re)discovered for reducing the tumor burden of the prostate cancer patient. Studies are under way in the laboratory to evaluate the efficacy of this compound in nude mice bearing LNCaP prostate cancer as explants. Results presented in this study strongly suggest that the beneficial effects curcumin begin where those of the conventional anti-androgen therapies end as it would kill both androgen-dependent and androgen-independent tumor

cells by forcing them to undergo apoptosis or apoptosis-like changes irrespective of whether these cells express one or multiple apoptosis suppressor proteins. Animal and human studies have indicated that systemic toxicity of curcumin is negligible.[26,27] Hence, in addition to its activity as a chemopreventive reagent, the potential therapeutic role of curcumin in advanced prostate cancers is worthy of further investigations and clinical trials. Or, at least, either dietary or systemically administered curcumin would synergize with the known anticancer agents for prostate cancer therapy such as taxol and estramustine to bring up a better kill at a lower anticancer drug concentrations, thus improving the quality of life of the prostate cancer patient.[58]

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