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Pro-Apoptotic and Immunomodulatory Activity of a Mycobacterial Cell Wall-DNA Complex Towards LNCaP Prostate Cancer Cells

S. Reader,* S. Ménard, B. Filion, Mario C. Filion,* and N.C. Phillips*

Bioniche Therapeutics Research Centre, Montréal, Québec, Canada

BACKGROUND. We have isolated a mycobacterial cell wall-DNA complex (MCC) possessing anti-cancer activity against bladder cancer cells. The anti-cancer activity of MCC appears to be due to two effects: a direct interaction with bladder cancer cells resulting in the induction of apoptosis and an indirect effect via the stimulation of monocytes and macrophages cytokine synthesis. In this study, the direct effect of MCC towards LNCaP cancer cells was evaluated.

METHODS. Inhibition of proliferation, cell cycle arrest and induction of apoptosis were evaluated in vitro using LNCaP cells treated with MCC. The synthesis of IL-12, GM-CSF, and TNF-α by LNCaP cells in response to MCC was also determined. Experiments were performed to gain insight into the mechanism of action of MCC towards LNCaP cells.

RESULTS. MCC caused a dose-dependent inhibition of the proliferation of LNCaP cells that was associated with cell cycle arrest at the G0/G1 phase. MCC-induced apoptosis of LNCaP cells was consistent with a mitochondrial pathway involving mitochondrial disruption, release of cytochrome c, and an increase in Bax protein levels leading to caspase-3 and -7 activation and cleavage of poly (ADP-ribose) polymerase and nuclear mitotic apparatus protein. Surprisingly, MCC also directly induced the synthesis of IL-12 and GM-CSF, but not TNF-α, by LNCaP cells.

CONCLUSIONS. MCC possesses the ability to directly induce apoptosis of LNCaP cells and to trigger the synthesis of IL-12 and GM-CSF by these cells, suggesting a potential role of MCC for the treatment of prostate cancer. *Prostate 49: 155–165, 2001.* © 2001 Wiley-Liss, Inc.

KEY WORDS: **apoptosis; caspase; mitochondria; interleukin-12; mycobacteria**

INTRODUCTION

Live mycobacteria or cell-wall skeleton prepared from mycobacteria has been shown to be effective in the treatment of a wide range of cancers [1–3]. The best known and extensively studied mycobacterial anti-cancer therapy is live bacillus Calmette-Guérin (BCG), an attenuated form of *Mycobacterium bovis*. Intravesical administration of live BCG is considered to be an effective treatment for transitional carcinoma of the bladder [4]. Additionally, a nonviable mycobacterial cell-wall extract derived from *Mycobacterium phlei* (*M. phlei*) has been shown to reduce cancer burden in orthotopic and heterotopic murine bladder cancer [5], and in patients with carcinoma in situ of the bladder [6].

The treatment of prostate cancer has been attempted using live mycobacteria or extracts from mycobacteria. BCG does not appear to be appropriate for the treatment of prostate cancer because of the development of systemic granulomatous lesions following intraprostatic administration of the live organism [7,8] or of nonviable extracts [9]. A mycobacterial cell-wall extract formulation derived from *M. phlei* has been found to reduce the tumor growth of the Dunning prostatic adenocarcinoma in rats [10]. We have

*Correspondence to: S. Reader, M.C. Filion, N.C. Phillips, Bioniche Therapeutics Research Centre, 6100 Royalmount, Montréal, Québec, Canada. H4P 2R2. E-mail: mario.filion@bioniche.com

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recently isolated a mycobacterial cell-wall complex composition from *M. phlei*, where mycobacterial DNA in the form of short oligonucleotides is preserved and complexed to the cell-wall (MCC) [11]. MCC possesses anti-cancer activity against bladder cancer cells [11]. The mycobacterial DNA associated with the cell-wall has been found to be essential for the anti-cancer activity of MCC [11,12]. This mycobacterial cell-wall composition, unlike live BCG or to non-viable BCG extracts, does not appear to induce granuloma or prostatitis when injected into the prostate of dogs (unpublished data). We have found that the anti-cancer mechanism of MCC towards bladder cancer cells appears to be different to that of live BCG. MCC is able to directly kill bladder cancer cells by inducing apoptosis [11], while BCG has been shown to reduce tumor growth by inducing a wide range of cytokines [13]. In addition to its pro-apoptotic activity, MCC has been found to induce the synthesis of cytokines by monocytes and macrophages, but fails to induce cytokine synthesis by bladder cancer cells [11,12].

In this study, we have evaluated the direct effect of MCC towards prostate cancer cells using the human prostate cancer cell line LNCaP, the most widely used in vitro model of prostate cancer [14]. Experiments were also performed to gain insight into the mechanism of action of MCC against these cells. We report herein that MCC inhibits LNCaP cell proliferation, and that this inhibition is associated with cell cycle arrest at the G0/G1 phase. We found that MCC-induced apoptosis of LNCaP cells is associated with increase levels of pro-apoptotic Bax protein, mitochondrial membrane potential ($\Delta\Psi_m$) disruption, release of cytochrome c from mitochondria, proteolytic activation of caspase-3 and -7, the cleavage of poly (ADP-ribose) polymerase (PARP) and the release of nuclear mitotic apparatus (NuMA) protein fragment. Surprisingly, we found that LNCaP cells were also able to synthesis IL-12 and GM-CSF, two potent anti-cancer cytokines, in response to MCC treatment.

MATERIALS AND METHODS

Antibodies and Other Reagents

Dimethylthiazoldiphenyltetrazolium bromide (MTT), RNase A, proteinase K, aprotinin, Hoescht 33258, and *E. coli* lipopolysaccharide (LPS; *E. coli* 011:B4) were from Sigma-Aldrich Canada (Oakville, ON, Canada). Leupeptin was from Roche Diagnostics (Laval, QC, Canada). Cycle TEST^{TM} plus DNA reagent kit was from Becton Dickinson (San Jose, CA). Nuclear mitotic apparatus protein (NuMA) immunoassay kit was from Oncogene Research Products (Cambridge, MA). 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) and anti-cytochrome oxidase subunit IV (clone 7H8.2C12) were from Molecular Probes, Inc. (Eugene, OR). Anti-cytochrome c (clone 7H8.2C12), anti-Bax (clone 6A7), anti-caspase-3 (rabbit polyclonal), anti-caspase-7 (clone B94-1), phycoerythrin (PE)-conjugated anti-active caspase-3 (rabbit polyclonal) and Ac-DEVD-CHO caspase-3 inhibitor were from BD Pharmigen (San Diego, CA). Fluorescein isothiocyanate (FITC)-conjugated anti-cleaved PARP antibody was from BioSource (Camarillo, CA).

Cell Culture and Treatment

LNCaP and PC3 prostate cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). PrEC, normal prostate epithelial cells, were obtained from Clonetics (Walkersville, MD). LNCaP and PC3 cells were maintained in RPMI 1640 medium, supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum, 1 mM Na pyruvate and 50 μg/ml gentamicin (all from Sigma-Aldrich Canada) in an atmosphere of 5% CO_2 at 37°C. PrEC cells were maintained in PrEGM medium in an atmosphere of 5% CO_2 at 37°C (Clonetics). Cells were seeded at 1×10^5 cells/ml medium in 6-well flat-bottomed tissue culture plates and incubated with PBS (used as the vehicle for MCC) or MCC for the indicated time period.

Proliferation Assays

After 48 hr treatment with MCC, cellular proliferation of LNCaP, PC3 and PrEC cells was measured by the reduction of MTT as described in [15]. Briefly, 100 μl of MTT stock solution (5 mg/ml in PBS) was added to the wells and the incubation continued at 37°C, 5% CO_2 for 4 hr. One ml of acidified isopropanol (isopropanol containing 0.04 M HCl) was then added, and solubilized formazan was measured at a wavelength of 570 nm.

Cytokine Analysis

After 48 hr of treatment with MCC, IL-12, GM-CSF, and TNF-α synthesis were examined in LNCaP cells by ELISA (BioSource). The ELISA sensitivity was 1 pg/ml for IL-12, TNF-α, and GM-CSF.

Cell Cycle Analysis

Cell cycle analysis of LNCaP cells was carried out by flow cytometry at the indicated times using the Cycle TEST^{TM} plus DNA reagent kit according to the manufacturer's instructions. Flow cytometry was carried out on a FACSCalibur (Becton Dickinson) using CELLQuest software (Becton Dickinson), and the cell cycle analyzed using Modfit LT software (Verity Software House, Inc., Topsham, ME).

Analysis of Apoptosis

Chromatin condensation and DNA fragmentation associated with apoptosis were examined in LNCaP cells by fluorescence microscopy. After 48 hr exposure to 100 and 300 μg/ml MCC, cells were washed twice with PBS and stained with 50 μl of 200 μM Hoechst 33258 for 20 min at room temperature. Chromatin condensation and nuclear fragmentation were visualized by fluorescence microscopy and photographs were taken. Apoptosis was also demonstrated by the solubilization and release of NuMA protein fragments. NuMA protein fragments released from MCC-treated or untreated LNCaP cells were analyzed after 48 hr of incubation. Supernatants were assayed for NuMA protein fragments by ELISA according to the manufacturer's instructions. For caspase inhibition experiments, LNCaP cells were treated with the caspase-3 inhibitor Ac-DEVD-CHO (50 μg/ml) for 12 hr prior to the addition of MCC.

Cytosolic Extracts and Cell Lysates Preparation

Cytosolic extracts free of mitochondria were prepared as described in [16]. Briefly, LNCaP cells were incubated with MCC. The cells were detached with PBS-1mM EDTA and washed three times with PBS, pH 7.2. The cell pellets were resuspended in lysis buffer (10 mM HEPES, pH 7.5, containing 5 mM $MgCl_2$, 1 mM dithiothreitol, 1.5 nM aprotinin, 10 nM leupeptin, and 2.5 μM Na orthovanadate) and passed several times through a 26-gauge needle fitted to a syringe. The lysate was centrifugated at 12,500 $\times g$ for 5 min at 4°C to pellet the nuclei and mitochondria. The supernatant was used as the cytosolic extract. To verify the absence of mitochondria in the cytosolic fraction, cytosolic and mitochondrial extracts were Western-blotted with an anti-mitochondrial cytochrome oxidase antibody. For Bax analysis, whole cell extracts were prepared by resuspending cell pellets in lysis buffer and then lysing the cells by sonication.

Immunoblotting

Protein content was determinated by the method of Bradford [17]. For each sample, 50 μg of protein was mixed with Laemmli buffer [18] shaken and boiled for 4 min. For cytochrome c, Bax and caspase analysis, samples were electrophoresed on a 17% SDS–polyacrylamide gel (SDS–PAGE) at a constant voltage of 100 V for approximately 1.5 hr. Separated proteins were then electroblotted onto a PVDF membrane. Equal protein loading was verified by Ponceau red staining of the membrane. The membranes were then blocked overnight at 4°C with Tris-buffered saline (2 mM Tris-HCl, pH 7.6, containing 13.7 mM NaCl, and 1% v/v Tween 20; TBST) containing 5% w/v non-fat dried milk. The membranes were incubated with the appropriate antibodies and the proteins were detected with a sheep anti-mouse IgG conjugated to horseradish peroxidase for caspase-7, cytochrome c, cytochrome oxidase and Bax, and with a donkey anti-rabbit IgG conjugated to horseradish peroxidase for caspase-3. The blots were then developed using an enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech, Baie d'Urfé, QC, Canada). For densitometric analysis, bands were analyzed using 1D Main software (Advanced American Biotechnology, Fullerton, CA).

Analysis of Active Form of Caspase-3 and Cleaved PARP by Flow Cytometry

For flow cytometric analysis of active caspase-3 and cleaved PARP, LNCaP cells treated with MCC were washed twice with PBS and fixed in cold 0.25% paraformaldehyde for 30 min at 4°C. After washing several times with PBS-1% FBS, the fixed cells were permeabilized with PBS containing 0.05% Tween 20 and 1% FBS for 15 min at 37°C. Cells were washed twice with PBS-1% FBS and labeled with PE-conjugated anti-active caspase-3 or FITC-conjugated anti-cleaved PARP antibodies for 30 min at 4°C. After washing twice with PBS-1% FBS, cellular fluorescence was then determined. Flow cytometry and data analysis were carried out using a FACSCalibur using CELLQuest software.

Mitochondrial Membrane Potential Determination

Mitochondial membrane potential ($\Delta\Psi_m$) in LNCaP cells was monitored by measurement of $DiOC_6(3)$ fluorescence using flow cytometry. After incubation with MCC, cells were detached from the tissue culture wells with ice-cold PBS containing 1mM EDTA, pH 7.2. The cells were washed 2 times with PBS and were loaded with 40 nM $DiOC_6(3)$ in PBS-1% FBS for 30 min at 37°C. Cellular fluorescence was then determined. Flow cytometry and data analysis were carried out using a FACSCalibur using CELLQuest software.

Statistical Analysis

Statistical analysis of differences between groups was performed using a paired *t*-test (GraphPad Prism™ software, GraphPad Software Inc., San Diego, CA).

RESULTS

Inhibition of Cell Proliferation of Normal Prostate Epithelial Cells and Prostate Cancer Cells

We initially determined whether MCC was able to inhibit cellular proliferation of normal prostate

epithelial cells (PrEC cells) and prostate cancer cells (LNCaP and PC3 cells) using an MTT reduction assay. As shown in Figure 1A, MCC inhibited the proliferation of LNCaP and PC3 cells in a concentration-dependent manner after 48 hr of treatment. MCC had little if any effect on PrEC cells. The mechanism(s) underlying the anti-proliferative activity of MCC, were further investigated by using LNCaP androgen-dependent prostate cancer cells, originally derived from a lymph node of a patient with metastatic prostate cancer.

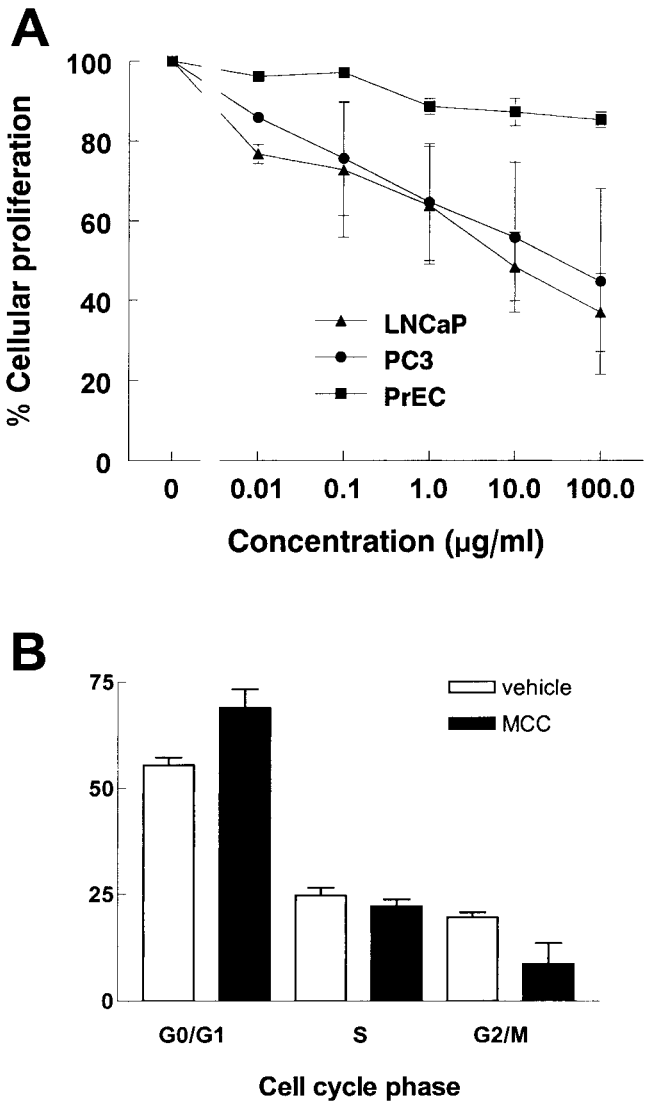


Fig. 1. Inhibition of cellular proliferation and cell cycle arrest. **A:** Cellular proliferation was measured by determination of MTT reduction as described in the Materials and Methods section. LNCaP, PC3, and PrEC cells were incubated in the presence of different concentrations of MCC for 48 hr. The results are the mean±SD of 3 independent experiments. **B:** LNCaP cells were incubated in the presence of 300 μg/ml of MCC for 48 hr. Cell cycle phases of LNCaP cells were determined as described in the Materials and Methods section. Results are expressed as the percentage of cells present in each phase. Accumulation of cells in G0/GI, S, and G2/M phases of the cell cycle were analyzed using Modfit LT software. The results shown are the mean±SD of 3 independent experiments. *Significantly different from controls ($P < 0.05$).

We next examined the effect of MCC on cell cycle progression of LNCaP cells by flow cytometry. When LNCaP cells were treated with 300 μg/ml of MCC for 48 hr, a significant increase in the number of cells in the G0/G1 phase of the cell cycle was observed ($P = 0.038$) in conjunction with a decrease in the number of cells in the G2/M phase (Fig. 1B). The percentage of cells in the S phase was almost unchanged after MCC treatment, indicating that MCC prevents G0/G1 LNCaP cells from entering the S phase of the cell cycle, as well as preventing their exit from S phase into G2/M.

MCC-Induced Apoptosis of LNCaP Cells

One of the mechanisms whereby MCC inhibits proliferation of prostate cancer cells could be through the induction of apoptosis. Apoptosis is associated with distinctive morphological changes that include condensation of nuclear chromatin, cell shrinkage, nuclear disintegration, plasma membrane blebbing, and the formation of membrane-bound apoptotic bodies [19]. Chromatin condensation and DNA fragmentation associated with apoptosis were evaluated by nuclear DNA staining with Hoescht 33258. In comparison with control cells (Fig. 2A), LNCaP cells incubated with 100 and 300 μg/ml of MCC were positive for Hoechst 33258 staining (Fig. 2B,C, respectively), indicative of cell death by apoptosis. At lower concentrations (1 and 10 μg/ml), MCC did not induce apoptosis (data not shown). Since no apoptosis was observed under these conditions, these concentrations were omitted in the following experiments.

The chromatin condensation detected by nuclear staining fluorescence was also accompanied by the solubilization and release of NuMA protein fragments into the medium. NuMA protein is a component of the nuclear mitotic apparatus, which may play a structural role in the architecture of the interphase nucleus [20]. During apoptosis NuMA protein is redistributed within the nucleus and is proteolysed [20]. As shown in Figure 3, MCC treatment of LNCaP cells caused a release of NuMA protein fragments into the medium after 48 hr of treatment in a concentration-dependent manner.

Effect of Ac-DEVD-CHO on MCC-induced NuMA Protein Fragments Released From LNCaP Cells

The role of caspases in MCC-induced apoptosis in LNCaP cells was examined using the caspase inhibitor

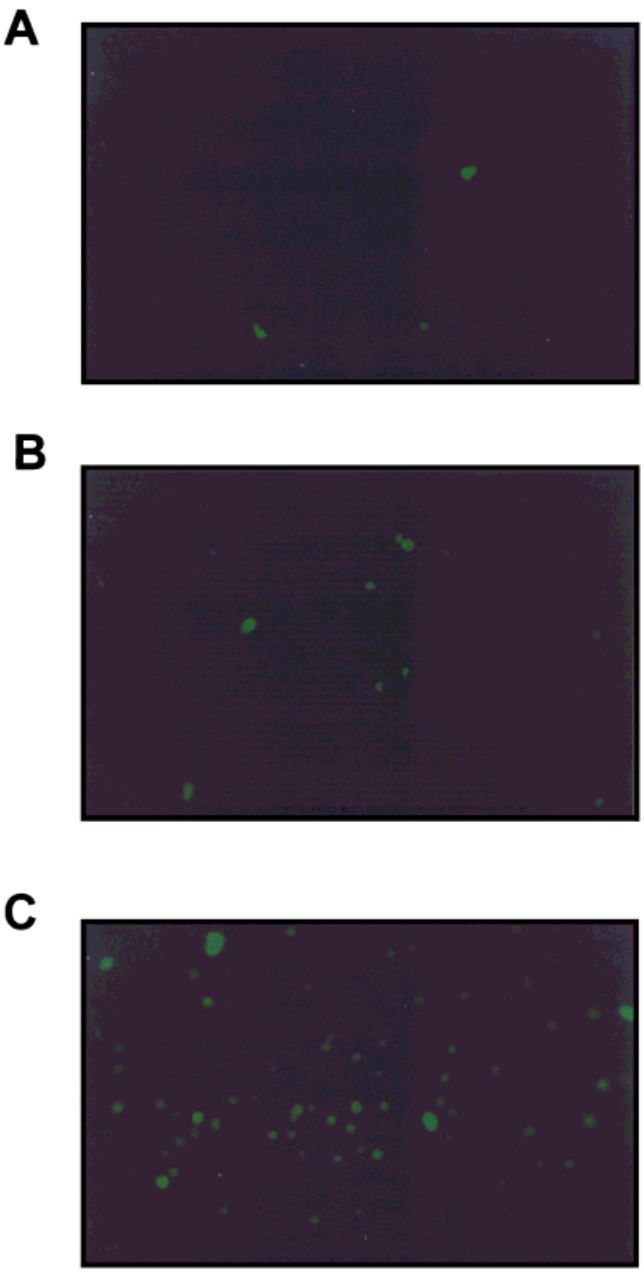


Fig. 2. Apoptosis of LNCaP cells induced by MCC as detected by nuclear DNA staining analysis. LNCaP cells were treated without (**A**) or with 100 μg/ml (**B**) or 300 μg/ml (**C**) of MCC for 48 hr. The cells were directly labeled with Hoechst 33258 as described in the Materials and Methods section. Images were collected using a Nikon fluorescence microscope equipped with a UV light source. Results represent one of three independent experiments. [This figure can be viewed in color online at www.interscience.wiley.com.]

Ac-DEVD-CHO. Although Ac-DEVD-CHO is primarily a caspase-3 inhibitor, it can also block other active caspases such as caspase-6, -7, and -8 [21]. The results show that the release of NuMA protein fragments induced by MCC was significantly reduced following Ac-DEVD-CHO treatment (Fig. 3) ($P = 0.0018$) demonstrating that the caspase cascade plays an important role in the execution process of MCC-induced apoptosis.

MCC-Induced Caspase Activation and PARP Cleavage in LNCaP Cells

To further investigate the role of caspases in MCC-induced apoptosis, we examined the proteolytic activation of procaspase-3 and -7 in LNCaP cells by Western blot and flow cytometric analysis. As shown in Figure 4A, procaspase-3 was cleaved and processed into its active form during the course of MCC-induced apoptosis of LNCaP cells. This was demonstrated by the depletion of the M_r 35,000 band (p35), representing procaspase-3 protein, accompanied by the appearance of the M_r 14,000 band (p14), representing the active subunit that contains the antibody epitope.

The proteolytic activation of procaspase-3 by MCC was confirmed by flow cytometric analysis using PE-conjugated anti-active caspase-3 antibody. Incubation of LNCaP cells with 100 and 300 μg/ml MCC for 48 hr resulted in an increase of fluorescence in LNCaP cells (Fig. 4B). This increase in fluorescence confirmed the presence of the active form of caspase-3 in MCC-treated LNCaP cells.

MCC treatment also modified the procaspase-7 content of LNCaP cells. Western blot analysis showed a significant reduction of the M_r 30,000 (p30) band corresponding to procaspase-7 protein in LNCaP cells, although the active caspase-7 subunit, p20, was not detectable (Fig. 4C). Densitometric analysis showed that after 48 hr treatment with 100 and 300 μg/ml of MCC, the level of procaspase-7 in LNCaP cells decreased by 55 and 65% respectively.

The cleavage of procaspase-3 and procaspase-7 following MCC treatment was accompanied by a reduction in PARP content of LNCaP cells. PARP, a repair enzyme, is a target protein for caspase-3 and caspase-7. The presence of cleaved PARP was detected by flow cytometry using an FITC-conjugated anti-cleaved PARP antibody. Flow cytometric analysis showed an increase of fluorescence, associated with the presence of cleaved PARP in LNCaP cells treated with 100 and 300 μg/ml of MCC (Fig. 4D). These results indicate that caspase-3 and caspase-7 are downstream effectors of MCC-induced apoptosis in LNCaP cells.

MCC-Induced Mitochondrial Membrane Disruption and Cytochrome c Release in LNCaP Cells

Substantial evidence has implicated the mitochondrion in the regulation of apoptosis [22,23]. To examine the possibility that MCC mediates its pro-apoptotic activity against LNCaP cells by effecting

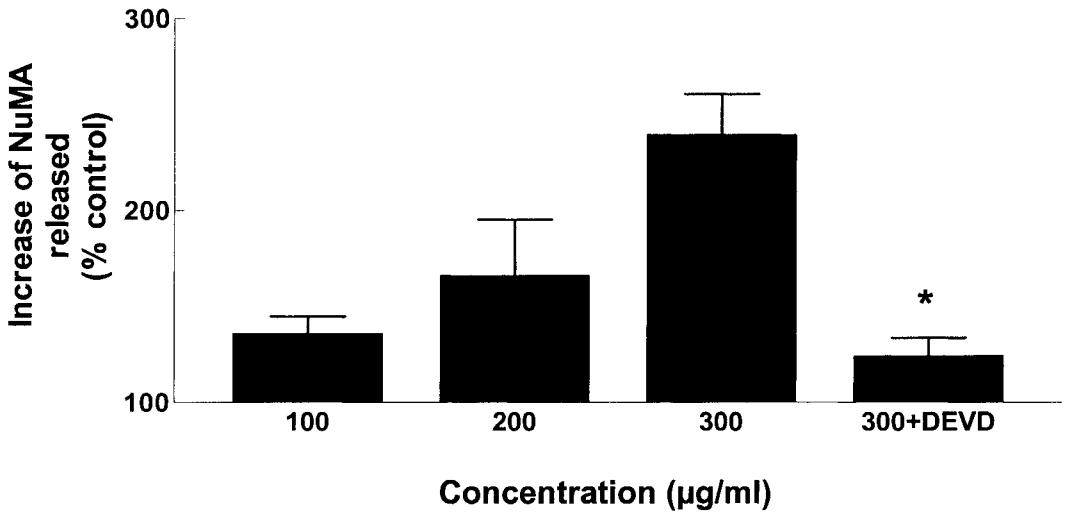


Fig. 3. Apoptosis of LNCaP cells by MCC as detected by NuMA protein fragments release. LNCaP cells were incubated in the presence of different concentrations of MCC. The release of NuMA protein fragments after 48 hr was determined by ELISA as described in the Materials and Methods section. For caspase inhibition experiments, LNCaP cells were treated with the caspase-3 inhibitor Ac-DEVD-CHO (50 μg/ml) for 12 hr and subsequently treated with 300 μg/ml of MCC for 48 hr. Results are expressed as the % increase in NuMA protein fragments release compared to untreated cells. The results shown are the mean±SD of 3 independent experiments. *Significantly different from cells treated with 300 μg/ml MCC ($P < 0.005$).

mitochondria, we firstly examined its action on $\Delta\Psi_m$ by flow cytometry using the fluorescent probe $DiOC_6(3)$. Mitochondrial hyperpolarization causes an increase in $DiOC_6(3)$ fluorescence emission, whereas a Ψ_m dissipation leads to a decrease in fluorescence. Figure 5 shows representative histogram overlays of Ψ_m after treatment of LNCaP cells with MCC. After 24 hr MCC treatment, cells stained with $DiOC_6(3)$ did not show any significant changes in $\Delta\Psi_m$. However, we found a dramatic change in their fluorescence pattern after 48 hr incubation in the presence of MCC (Fig. 5A). Thirty percent of LNCaP cells showed an increase in $DiOC_6(3)$ fluorescence, indicating mitochondrial hyperpolarization, and 70% of the cells had depolarized mitochondria after 48 hr of treatment with 300 μg/ml of MCC (Fig. 5A).

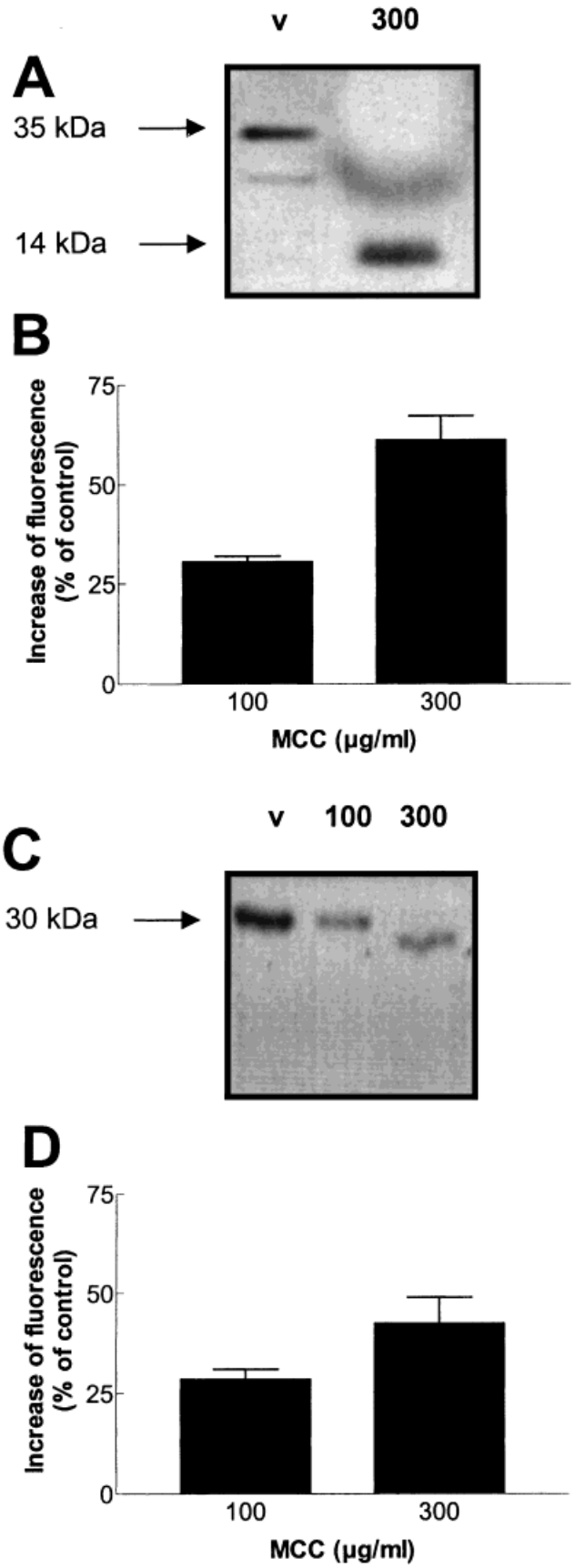
Changes in $\Delta\Psi_m$ and mitochondrial membrane permeability are two important events in the release of cytochrome c [22,23]. In a number of cases, cytochrome c release from the mitochondria into the cytosol has been identified as an initiator of the apoptotic cascade [24,25]. To examine this possibility, we studied the accumulation of cytochrome c in the cytosol of MCC-treated cells by Western blot. As shown in Figure 5B, MCC treatment caused an accumulation of cytochrome c in the cytosol of LNCaP cells. Anti-cytochrome oxidase was used to verify if the presence of cytochrome c in the cytosolic fraction was the result of mitochondrial contamination. The very small amount of cytochrome oxidase detected confirmed that the cytosolic fraction was essentially mitochondria-free (data not shown). The results indicate that disruption of $\Delta\Psi_m$, accompanied by cytochrome c release, is involved in the cascade of events associated with MCC-induced apoptosis.

MCC-Induced Increase of Bax Protein in LNCaP Cells

We next examined whether MCC treatment is associated with a change in Bax protein levels. Bax is a pro-apoptotic member of the Bcl-2 family of proteins, capable of triggering apoptosis either directly through its effects on mitochondrial function or indirectly by counteracting the anti-apoptotic protein Bcl-2 [22,26–28]. The level of Bax protein in MCC-treated LNCaP cells was examined by Western blot analysis. The results showed that MCC treatment increases the level of Bax protein in LNCaP cells after 24 hr, as demonstrated by the presence of the M_r 20,000 band (p20) (Fig. 5C).

Induction of IL-12 and GM-CSF Synthesis in LNCaP Cells by MCC

IL-12 is an inducible cytokine that is predominately synthesized by B lymphocytes, macrophages and dendritic cells, and which possesses potent anti-cancer activity following systemic or local administration in a variety of malignancies [29]. We have previously shown that MCC is a potent inducer of IL-12 synthesis by monocytes and macrophages, but that MCC fails to induce the synthesis of this cytokine by bladder cancer



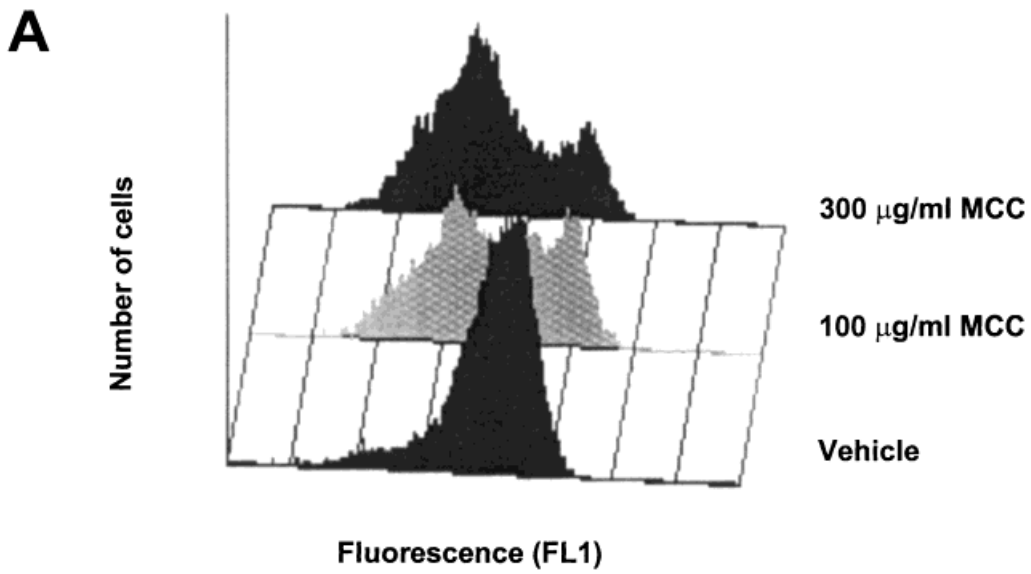
cells and by B and T lymphocytes [12,13]. We have therefore evaluated whether MCC could not only induce the synthesis of IL-12 by LNCaP cells, but also GM-CSF and TNF-α, two cytokines that can influence tumor growth outcome. As shown in Figure 6, MCC induces both IL-12 and GM-CSF synthesis by LNCaP cells. LPS, a potent immunomodulator capable of inducing the synthesis of these cytokines by monocytes and macrophages, was not able to induce their synthesis in LNCaP cells (data not shown). Maximum IL-12 and GM-CSF synthesis was obtained using a concentration of 10 µg/ml of MCC. MCC was not able to induce the synthesis of TNF-α by LNCaP cells in the concentration range of 1–100 µg/ml of MCC (Fig. 6).

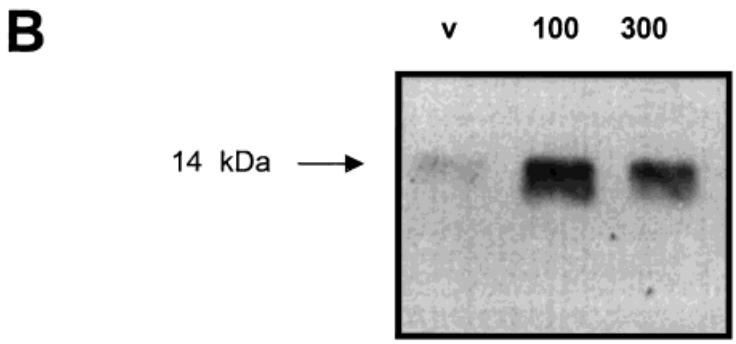
DISCUSSION

Novel strategies for the treatment of prostate cancer are directed towards the development of indirect immunomodulatory or direct apoptosis-inducing therapies [30,31]. In the present study, we have shown that MCC, a mycobacterial cell wall composition derived from *M. phlei*, possesses a direct anti-proliferative activity towards LNCaP cells that is associated with an increase in the number of cells in G0/G1 phase of the cell cycle. The presence of apoptosis in LNCaP cells following MCC treatment was demonstrated by typical DNA condensation observed on Hoescht 33258 staining (Fig. 2), and by the presence of NuMA protein (Fig. 3). Similar results regarding cell cycle arrest and apoptosis were obtained after MCC treatment of PC3 (unpublished data). MCC had little or no anti-proliferative activity against normal prostate epithelial cells.

Cytokines have been shown to exert both cytostatic and immunomodulatory effect on prostate cancer cells. TNF-α directly inhibits the proliferation of a number of prostate cancer cell lines [31], and modulates the expression of prostate-specific antigen, androgen receptor, and HLA Class I [32]. However, TNF-α can also promote angiogenesis in prostate cancer [33]. GM-CSF is capable of inducing systemic immune responses against prostate cancer. The

Fig. 4. Activation of procaspase-3, -7, and PARP in LNCaP cells induced by MCC. Western blot analysis of the cleavage of procaspase-3 (**A**) and the level of procaspase-7 (**C**) in LNCaP cells treated with vehicle (v), 100 or 300 µg/ml of MCC for 48 hr. Proteins were extracted as described in the Materials and Methods section. PVDF membranes containing 50 µg cell extract protein/lane were blotted with antibodies to the indicated proteins as described in the Materials and Methods section. Flow cytometric analysis of cleaved-caspase-3 (**B**), and cleaved-PARP (**D**) in LNCaP cells treated with MCC for 48 hr. Cells were prepared as described in the Materials and Methods section. Flow cytometry and data analysis were carried out using a FACSCalibur using CELLQuest software. Similar results were obtained in three independent experiments.





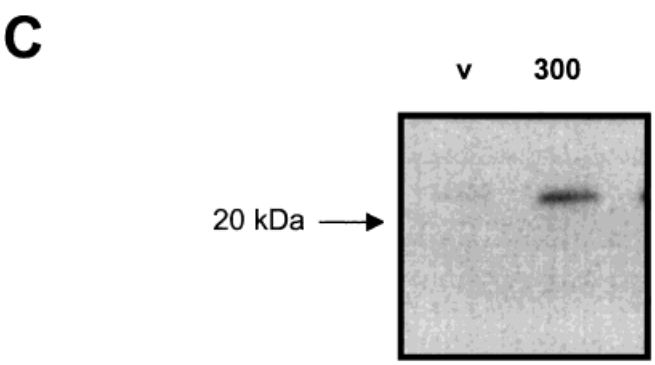


Fig. 5. Mitochondrial membrane disruption, release of cytochrome c and bax elevation in LNCaP cells induced by MCC. **A:** Changes in $\Delta\Psi_m$ of LNCaP cells were analyzed by flow cytometry. LNCaP cells were treated with vehicle, 100 and 300 μg/ml of MCC for 48 hr. After MCC treatment, cells were loaded with $DiOC_6(3)$ as described in the Materials and Methods section. Mitochondrial hyperpolarization causes an increase in $DiOC_6(3)$ fluorescence emission, whereas a $\Delta\Psi_m$ dissipation leads to a decrease in the fluorescence. Representative histogram overlays show the $\Delta\Psi_m$ profiles after incubation of LNCaP cells with MCC. Each concentration represents the analysis of 5,000 events. **B:** Cytochrome c release. LNCaP cells were incubated with vehicle (v), 100 or 300 μg/ml of MCC. After 48 hr, cellular extracts were prepared as described in the Materials and Methods section, and cytosolic cytochrome c determined by Western blot analysis. The 14 kDa band corresponds to cytochrome c protein. **C:** Bax levels. LNCaP cells were treated with MCC as described above. Total cellular extracts were prepared as described in the Materials and Methods section, and Bax determined by Western blot analysis. The 20 kDa band corresponds to Bax protein. Similar results were obtained for (A), (B) and (C) in 3 independent experiments.

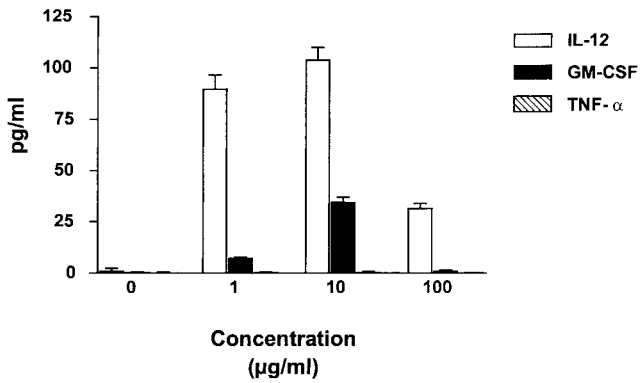


Fig. 6. Induction of IL-12 and GM-CSF in LNCaP cells by MCC. LNCaP cells were incubated in the presence of different concentrations of MCC for 48 hr. IL-12, GM-CSF, and TNF-α released into the supernatant were determined as described in the Materials and Methods section. The results are the mean±SD of 3 independent experiments.

anti-tumor activity of recombinant GM-CSF or cancer cells engineered to secrete GM-CSF has been confirmed in a number of clinical trials [34,35]. Furthermore, GM-CSF can inhibit angiogenesis in prostate cancer by the induction of the synthesis of the anti-angiogenic protein plasminogen activator inhibitor type 2 [33]. IL-12 also has anti-tumor activity following its systemic or local administration against a variety of malignancies including prostate cancer cells [36,37]. IL-12 appears to exert its anti-tumor activity by stimulating natural killer cells, T-lymphocytes, enhancing cytokine expression and blocking angiogenesis [29]. To our surprise, we have found that MCC was able to directly induce the synthesis of IL-12 and GM-CSF by LNCaP cells, but did not induce the synthesis of TNF-α in the concentration range of 1–100 μg/ml. LNCaP cells have been found to produce pro-inflammatory molecules in response to various stimuli (hypoxia, anticancer drugs and thalidomide) [38,39], but to our knowledge, it has not been demonstrated that these cells are able to produce IL-12 and GM-CSF in response to an anti-cancer agent. It has been shown recently that the established human prostate cancer cell lines PC-3, DU-145, and TSU, and normal human prostate epithelial cells in culture can produce cytokines such as IL-8, TNF-α, and GM-CSF [40,41]. Therefore, MCC appears to have a direct immunomodulary activity on LNCaP cells, as evidenced by the induction of IL-12 and GM-CSF synthesis. Experiments are underway to determine whether MCC is able to induce the synthesis of cytokines by other prostate cancer cell lines or by normal epithelial cells.

The concentration of MCC used with LNCaP cells appears to be important. We have found that MCC at a concentration of 10 μg/ml induces the synthesis of IL-12 and GM-CSF, but not inhibition of proliferation or induction of apoptosis in LNCaP cells, while at a concentration of 100 μg/ml MCC shows both immunomodulatory and pro-apoptotic activity. The ability of MCC to induce apoptosis of LNCaP cells at 100 μg/ml may explain why at this concentration less IL-12 and GM-CSF were detected in the supernatant. GM-CSF has been reported to stimulate the growth of LNCaP cells [42]. We have not observed a stimulation of the proliferation of LNCaP cells in the presence of MCC. This could be explained by the fact that MCC not only induced the synthesis of GM-CSF but arrested the cell cycle and triggered apoptosis. The induction of cytokine synthesis in vivo may make a significant contribution to the host response against prostate cancer cells.

Treatment of prostate cancer by BCG is contraindicated due to the formation of severe granuloma and other side effects [7–9]. MCC does not appear to induce granuloma following intra-prostatic administration in dogs (unpublished data). This could be explained by the fact that MCC has the capacity to induce apoptosis. Apoptosis allows the clearance of dead cancer cells from living tissues without causing inflammation [43]. Live BCG does not have the capacity to directly induce apoptosis of bladder cancer cells [44,45]. Heat-killed *Mycobacterium vaccae,* used as an adjuvant for tumor cell vaccination,also has no direct activity on prostate tumor growth [46]. The pro-apoptotic activity of MCC towards bladder cancer cells [12] and prostate cancer cells (this present study) may be an important factor in its overall anti-cancer activity and may distinguish it from other ''mycobacteria'' treatment.

The apoptosis-inducing mechanism of MCC towards LNCaP cells was investigated in this study. Apoptosis induced by MCC was significantly inhibited by the caspase inhibitor Ac-DEVD-CHO, indicating the involvement of caspases in the execution of apoptosis. The implication of caspases in MCC-induced apoptosis was further confirmed by the presence of the proteolytically activated form of caspase-3, the degradation of procaspase-7 and by the presence of cleaved PARP and NuMA protein fragments. These results suggest that caspase-3 and -7 participate in the execution phase of apoptosis induced by MCC.

To gain further insights into the pathway(s) of MCC-induced apoptosis, we investigated the possibility that MCC-induces apoptosis by perturbing the Ψ_m and/or releasing mitochondrial cytochrome c. There is emerging evidence that changes of $\Delta\Psi_m$ and mitochondrial membrane permeability are two important events in the pro-apoptotic signalling cascade leading to the release of cytochrome c. The precise

mechanism by which cytochrome c is released from mitochondria during apoptosis remains unclear. Recently, Tsujimoto and collaborators [47] have shown that Bax and Bak, two pro-apoptotic members of the Bcl-2 family proteins, bind to the voltage-dependent anion channel (VDAC) and accelelerate channel opening to allow the passage of cytochrome c into the cytosol, while anti-apoptotic Bcl-x_L, through direct binding, closes the VDAC [48]. The opening of mitochondrial permeability transition pores, which results in a reduction of $\Delta\Psi_m$, also could be responsible for cytochrome c release [49,50]. Finally, mitochondrial swelling and rupture of the outer membrane, independent of loss of $\Delta\Psi_m$, can lead to a leakage of cytochrome c [51,52]. Ours results show that MCC causes $\Delta\Psi_m$ disruption and cytochrome c release into the cytosol of MCC-treated LNCaP cells. Taken together, these results indicate that $\Delta\Psi_m$ disruption caused by MCC might be responsible for the release of cytochrome c from mitochondria into the cytosol. Hence, our data suggest that MCC is able to induce apoptosis by a mitochondrial pathway culminating in the release of cytochrome c and the following activation of caspases.

It has been previously demonstrated that the addition of Bax to isolated mitochondria induces cytochrome c release [53]. Following an apoptotic signal, Bax translocates from the cytosol to the mitochondria, undergoes a conformational change, oligomerizes and inserts into the outer membrane of the mitochondrion. These events are followed by the release of cytochrome c into the cytosol [53]. Our results demonstrate an increase of Bax levels in LNCaP cells following MCC treatment. These results suggest that MCC-mediated cytochrome c release and pro-apoptotic activity towards LNCaP cells may involve the participation of this protein. The increased levels of Bax following MCC treatment suggests that de novo synthesis, as well as redistribution, plays a significant role.

In conclusion, our data shows that in the presence of MCC, LNCaP cells (1) produce IL-12 and GM-CSF, and (2) undergo apoptosis consistent with a mitochondrial to caspase activation pathway. The combination of chemotherapeutic-like (apoptosis) and immunotherapeutic (cytokine induction) activities in the absence of immune effector cells may be important for the treatment of prostate cancer. We are presently evaluating the effectiveness of MCC in experimental prostate cancer models.

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