Susceptibility of the human LNCaP prostate cancer cells to the apoptotic effect of marina crystal minerals (MCM) in vitro

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Received February 5, 2009; Accepted March 26, 2009

DOI: 10.3892/or_00000419

Abstract. Prostate cancer is one of the most frequent cancers among men in the United States. In the current study, we examined the susceptibility of the human LNCaP prostate cancer cells to the apoptotic effect of marina crystal minerals (MCM), a crystallized mixture of minerals and trace elements from sea water, in vitro. Cancer cells were cultured with MCM at different concentrations (0-1000 μg/ml). At day 3, MCM exhibited a marked apoptotic effect on LNCaP cells as measured by propidium iodide (PI) and Giemsa-stained cytospin preparations. The apoptotic effect was dose-dependent: a 2-fold increase in the level of apoptosis over the control cells was observed at low concentrations of 50 μg/ml, maximal (3-fold) at a concentration of 500-1000 μg/ml. The mechanism by which MCM induces apoptosis was examined. Flow cytometry analysis demonstrated that mitochondrial membrane potential was significantly decreased post-treatment with MCM at low concentrations of 100 μg/ml. In addition, Western blot analysis showed that the level of anti-apoptotic molecule Bcl-2 in LNCaP cells decreased upon treatment with MCM. We conclude that MCM has an apoptotic effect toward human prostate cancer cells, which may suggest its broader potential as an innovative, non-toxic anti-cancer agent.

Introduction

Prostate cancer is the most common non-cutaneous cancer among males. Prostate-specific antigen (PSA) and digital rectal examination (DRE) are the 2 components necessary for a modern screening program. While prostate cancer can be a slow-growing cancer, thousands of men die of the disease each year. According to recent figures from the American Cancer Society, 220,900 new cases were diagnosed in 2003 and 28,900 men were predicted to die of prostate cancer. Prostate cancer is rarely diagnosed in men younger than 40 years and it is uncommon in men younger than 50 years. Prostate cancer is also found during autopsies performed following other causes of death. The rate of this latent- or autopsy-diagnosed cancer is much greater than that of clinical cancer. In fact, it may be as high as 80% by age 80 years. In addition, genetic studies suggest that a strong familial predisposition may be responsible for as many as 5-10% of prostate cancer cases (1,2). Most prostate cancers are adenocarcinomas (95%). Prior to metastasis, oncologists mainly rely on surgery, chemotherapy and radiation therapy for cancer treatment of prostate cancers. The destruction of cells by chemotherapy and radiation therapy is indiscriminate with serious side effects. In addition to being toxic, both chemotherapy and radiation therapy are also immune-suppressive, mutagenic and carcinogenic (3-6). Hormone therapy is also commonly used to treat prostate cancer. Most clinical trials to date have evaluated flutamide or nilutamide for the treatment of advanced prostate cancer. Results showed that these drugs resulted in a survival increase of only 8% at 5 years (7).

In the field of prostate cancer therapeutics, it is known that some cancers respond well to specific chemotherapy while others do not. Age and health status play a significant role in determining treatment regimens and the toxic effect of chemotherapy may exclude this treatment as an option for some patients. For prostate cancer that metastasizes, there is no cure and treatment is mostly palliative. Such considerations emphasize the need to identify new compounds as possible therapeutic agents for human prostate cancer with minimal or no side effects.

Our recent studies show that marina crystal minerals (MCM), a crystallized mixture of minerals and trace elements from sea water, has an apoptotic effect against human metastatic breast cancer cells (8) and an overall immune modulatory effect (9). In the current study, we investigated whether MCM extends its apoptotic effect also against prostate cancer cells. Results demonstrated that MCM inhibits the growth of prostate cancer cells (LNCaP) by a mechanism that involves down-regulation of the anti-apoptotic molecule Bcl-2. This observation coupled with MCM’s ability to enhance human NK cell activity suggests that MCM could be used as a new potent anti-cancer agent.
Materials and methods

Tumor cell line. Human prostate cancer cells (LNCaP) were used in the present study. Cells were purchased from American Type Culture Collection (ATCC), Manassas VA, USA. Tumor cells were maintained in our laboratory by serial passage at a starting density of 3x10^5 cells/ml in complete medium.

Complete medium (CM). CM consists of RPMI-1640 supplemented with 10 mg/ml glucose, 10% heat inactivated fetal calf serum and 100 units penicillin and 100 μg/ml streptomycin.

Marina crystal minerals (MCM). MCM is a crystallized mixture of minerals, trace elements and other active ingredients from sea water, which were originally separated from sodium chloride. MCM was dissolved in CM at various concentrations (0-1000 μg/ml). MCM was provided by the Foundation for Basic Research Institute of Oncology, Japan.

Growth of monolayer LNCaP in 8-well plates. A model assay system previously developed by us (10) was used to examine apoptosis of monolayer LNCaP following culture with MCM. This system allows us to examine apoptosis of adherent cancer cells. Cancer cells were allowed to grow in 8-well plates (26x33 mm each) (LUX Scientific Corp., Thousand Oaks, CA, USA) with a cover glass placed at the bottom of each well. Cancer cells (1x10^6 cells/ml) were pipetted into each well and allowed to adhere. After 2 h, the cells were washed once with 1 ml CM. To each well, MCM (500 μg/ml) was added and plates were incubated at 37°C and 5% CO2. At three days post-treatment, adherent cells were examined as follows: Cover glasses containing adherent cells were carefully removed, air-dried, and mounted on slides. Preparations were fixed in 100% methanol, air-dried, stained with 4% Giemsa stain (Sigma-Aldrich Corp., St. Louis, MO, USA) for 15 min and examined for percent apoptosis using a light microscope fitted with a 100X oil immersion objective (Nikon, Tokyo, Japan).

Detection of mitochondrial membrane potential (MMP). Variations of the MMP during apoptosis were studied using tetramethylrhodamine ethyl ester (TMRE, Molecular Probes, Eugene, OR, USA). Briefly, after treatment with MCM (100-500 μg/ml) for 24 h, 5x10^3 cells/ml were incubated with 50 nM TMRE for 30 min at 37°C. The cells were washed with PBS and analyzed with FACSscan flow cytometry (Becton-Dickinson). Western blot analysis. The expression of Bcl-2 was determined using Western blot analysis. Cancer cells were cultured with MCM (0-1000 μg/ml) for 3 days. The cells were harvested, washed with cold PBS [10 mmol/l (pH 7.4)], and lysed with ice-cold lysis buffer [50 mmol/l Tris-HCl, 150 mmol/l NaCl, 1 mmol/l EGTA, 1 mmol/l EDTA, 20 mmol/l NaF, 100 mmol/l Na3VO4, 1% NP40, 1 mmol/l phenyl-methylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin (pH 7.4)] for 30 min and centrifuged at 14,000 x g for 20 min at 4°C, as detailed previously (12). The supernatant was collected and used immediately. The protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. The cell lysate (75 μg) in lysis buffer were subjected to Western blot analysis by 4-12% SDS polyacrylamide gel electrophoresis using 1:500 anti-Bcl-2 antibody (BD Biosciences, San Jose, CA, USA) as primary antibody. The washed polyvinylidene fluoride (PVDF) membranes were incubated with a 1:2000 dilution of monoclonal secondary antibodies. The immunoreactive bands were visualized using an ECL Western blotting kit (Amersham, Buckinghamshire, UK). To verify equal protein loading and transfer, the blots were stripped and re-probed with a rabbit anti-β actin polyclonal antibody. Thereafter the same protocol was followed as detailed above.

Results

Flow cytometry analysis. To determine whether MCM exerts an apoptotic effect against human prostate cancer, LNCaP cancer cells were cultured with MCM at various concentrations (0-1000 μg/ml) for 3 days. The apoptotic effect of MCM was then analyzed using flow cytometry. Results showed that LNCaP cells demonstrated a remarkable apoptotic response to the apoptotic effect of MCM in a dose-dependent fashion (Fig. 1). A significant induction of apoptosis (a 2-fold increase over the control cells) was detected at low concentrations of MCM (50 μg/ml). The apoptotic effect of MCM was maximal (3-fold) at a concentration of 500 μg/ml and was maintained at the same level at higher concentrations of 1000 μg/ml.

Detection of apoptotic cells by morphological analysis. Apoptosis is morphologically defined by cell shrinkage, membrane blebbing and chromatin condensation. These criteria were used to identify apoptotic LNCaP cells in cytospin preparations stained with Giemsa. Cancer cells were treated for 3 days with MCM (250 and 500 μg/ml) and adherent cells were analyzed for percent apoptosis using microscopy of Giemsa-stained preparations. Results in Fig. 2 showed a significantly increased level of apoptosis in cancer cells following treatment with MCM as compared with control untreated cells. MCM treatment at concentrations of 250 and 500 μg/ml demonstrated 2-fold and 3-fold increase over control, untreated cells (P<0.01).

Effect of MCM on mitochondrial membrane potential. Treatment of LNCaP cells with MCM for 24 h resulted in a decrease in the mitochondrial polarization. Data in Fig. 3 demonstrate that the effect was significant at a concentration
Effect of MCM on Bcl-2 protein levels. The protein expression of the anti-apoptotic molecule Bcl-2 was examined in LNCaP cells post-treatment with MCM (0-1000 μg/ml). Fig. 4 depicts the results of Western blot analysis, showing that treatment of cancer cells with MCM for 3 days resulted in significant down-regulation of the anti-apoptotic protein Bcl-2 (P<0.001) as compared to control untreated cells.

Discussion

The results presented have demonstrated that marina crystal minerals (MCM) can be used as a potent apoptotic agent for the human prostate cancer cell line LNCaP in vitro. The mechanism by which MCM induces apoptosis in LNCaP cells was examined. Results demonstrated that the mitochondrial membrane potential (MMP) was significantly decreased following treatment with low concentrations (100-500 μg/ml) of MCM. Maintenance of the MMP is tightly regulated and essential for normal cell function. Permeabilization of the mitochondrial inner membrane leads to changes in MMP. This permeabilization results in the release of pro-apoptotic factors into the cytosol and subsequent initiation of apoptosis (13,14). The anti-apoptotic protein
Bcl-2 is localized to the mitochondrial inner membrane and appears to play an important role in the maintenance of the MMP. Western blot analysis showed that treatment of cancer cells with MCM for 3 days resulted in significant down-regulation of Bcl-2 (P<0.001) as compared to control untreated cells. Within the mitochondria are apoptotic factors (cytochrome c, Smac/DIABLO, Omi) that if released activate the executioners of apoptosis, the caspases. Bcl-2 blocks the release of these apoptotic factors. Down-regulation of Bcl-2 results in an increase in the release of caspases and other factors that induce apoptosis. In our recent study, we also showed that MCM has an apoptotic effect toward human metastatic breast cancer MDA-MB-231 cells and by a similar mechanism that involves down-regulation of Bcl-2.

MCM is isolated from the Orai-Oki Sea in the Ibaraji Prefecture, Japan, which contains the greatest concentration of plankton in the world. One ton of sea water produces 1 kg of sodium chloride-free MCM. Chemical analysis of the ingredients in sodium chloride-free MCM was conducted, and no harmful trace elements were detected. This analysis identified mineral and trace elements such as ferrate (Fe), copper (Cu), chromium-III (Cr-III), manganese (Mn), zinc (Zn) and selenium (Se) in MCM. Each of these trace elements plays an essential biological role in humans and many exert an apoptotic effect against cancer. For example, Cu²⁺ plays an important physiological role whose homeostasis is tightly regulated. Disruption of Cu²⁺ homeostasis has been linked to several serious disorders, including cancer (15-17). The link between cancer and copper homeostasis has been bolstered by the discovery that Cu²⁺ treatment can inhibit neoplastic growth and increase survival in rat and mouse models of cancer (18). Increased uptake of other trace elements, such as Se and Zn, has been suggested to decrease the incidence of cancer (19,20). In addition, proteins containing trace elements such as Se, Cu, Mn and Zn have been identified in the prostate and are proposed to function in apoptosis and redox processes (21). It is not clear whether the observed increased apoptotic effect by MCM against LNCaP cells could be attributed to down-regulation of Bcl-2.

Several previous studies have also characterized the apoptotic effect that several trace elements have in cancer cells. For example, zinc sulfate was shown to induce apoptosis of human metastatic breast cancer MDA-MB-231 cells in vitro. Disruption of Cu²⁺ homeostasis has been linked to several serious disorders, including cancer (15-17). The link between cancer and copper homeostasis has been bolstered by the discovery that Cu²⁺ treatment can inhibit neoplastic growth and increase survival in rat and mouse models of cancer (18). Increased uptake of other trace elements, such as Se and Zn, has been suggested to decrease the incidence of cancer (19,20). In addition, proteins containing trace elements such as Se, Cu, Mn and Zn have been identified in the prostate and are proposed to function in apoptosis and redox processes (21). It is not clear whether the observed increased apoptotic effect by MCM against LNCaP cells could be attributed to down-regulation of Bcl-2.

MCM showed no negative effect on the proliferation of peripheral blood mononuclear cells (9), indicating it has low toxicity toward normal cells.

Immunological stimulation is currently receiving attention as a major treatment modality in the management of cancer patients (26-28). Several types of immune cells, such as cytotoxic T lymphocytes (CTL) and natural killer (NK) cells have demonstrated anti-cancer activity and may represent a promising cell therapy for the treatment of prostate cancer. However, prostate cancer is able to evade CTL immune surveillance through frequent loss of MHC class I and II expression in cancer cells and tissues (29-33), indicating that attempts to activate T cells may not be a successful strategy for the treatment of prostate cancer. On the other hand, many attempts were made to enhance activity of NK cells and expand their population, which are suppressed in prostate cancer patients (34,35). These enhanced NK cells can then be used as effector cells for the treatment of prostate cancer (36-39). It is of great interest that MCM is a potent biological response modifier (BRM) that has the ability to enhance human NK cell activity in vivo and in vitro (9). Moreover, MCM was found to significantly increase interferon (IFN-γ) production (9), another important component of anti-cancer activity. Taken together, our data indicate that MCM possess anti-tumor activity and immunomodulatory properties and suggest its use as a new potent anti-cancer agent.

References