PectaSol-C Modified Citrus Pectin Induces Apoptosis and Inhibition of Proliferation in Human and Mouse Androgen-Dependent and -Independent Prostate Cancer Cells

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Abstract

Aim: To demonstrate the efficacy of PectaSol-C modified citrus pectin (MCP) on prostate cancer in vitro. Method: Cytotoxicity analysis of PectaSol-C was performed by MTT assay, as were parallel studies with the former brand version of MCP called PectaSol. Apoptosis and inhibition of cell growth were investigated by Western blotting. Results: Androgen-dependent and -independent human prostate cancer cell lines (LNCaP and PC3, respectively), androgen-dependent and -independent murine prostate cancer cell lines (CASP2.1 and CASP1.1, respectively), as well as noncancerous human benign prostate hyperplasia BPH-1 cell line, were used in the study. MTT assay revealed that 1.0% PectaSol exerted cytotoxicity on LNCaP, PC3, CASP2.1, CASP1.1, and BPH-1 cells for 4-day treatment by 48.0% ± 2.1%, 54.4% ± 0.3%, 15.4% ± 0.8%, 46.1% ± 1.7%, and 27.4% ± 1.6%, respectively; whereas 1.0% PectaSol-C showed cytotoxicity by 52.2% ± 1.8%, 48.2% ± 2.9%, 23.0% ± 2.6%, 49.0% ± 1.3%, and 26.8% ± 2.6%, respectively. Western blotting further confirmed that both MCPs inhibit MAP kinase activation, increase the expression level of its downstream target Bim, a pro-apoptotic protein, and induce the cleavage of Caspase-3 in PC3 and CASP1.1 prostate cancer cells. Conclusion: PectaSol MCP and PectaSol-C MCP can inhibit cell proliferation and apoptosis in prostate cancer cell lines. Our data suggested that 1.0% PectaSol-C can be used for further chemopreventive and chemotherapeutic analysis in vivo.

Keywords
prostate cancer, modified citrus pectin, Caspase-3, apoptosis, MAPK

Introduction

Dietary carbohydrates have been suggested to have various health-promoting properties.1-9 Pectin is a complex carbohydrate-soluble fiber. Although pectins are not digestible by humans, modified citrus pectin (MCP) is altered by decreasing its molecular weight and thus believed to increase its absorbability into the blood circulation. The results of in vivo animal and human clinical studies with oral administration of MCP, which are discussed below, suggest absorption into the circulatory system. The MCPs used in this study are composed of short slightly branched carbohydrate chains derived from the soluble albedo fraction of citrus fruit peels. MCP polysaccharides are derived from commercial pectin isolated from citrus fruits, which have a molecular weight of 100 000 to 200 000 Da. Modification via pH, temperature, and/or enzymatic degradation produces MCP that is much lower in molecular weight and less structurally complex, compared with the original citrus pectin. As a result, MCP dissolves readily in water and is believed to be better absorbed and utilized by the body than ordinary, long-chain pectin. In the United States, MCP is registered as a dietary supplement.

MCP is relatively rich in galactose and antagonizes a binding protein galectin-3 (Gal-3), which results in suppression of cancer metastasis.7,10 It has been shown that MCP can increase prostate-specific antigen (PSA) doubling time in 7 of 10 men (with biochemical recurrence of prostate cancer following local therapy) after taking MCP for 12 months, compared with before taking MCP.11 Moreover, oral intake of MCP can reduce lung metastases of rat prostate cancer cells in the Copenhagen rat.12 These studies are
indicative that MCP can act as a potent agent for the treatment of cancer.

The shorter polysaccharide units afford this type of MCP with its ability to access and bind tightly to galactose-binding lectins (galectins) on the surface of certain types of cancer cells. For metastasis to occur, cancerous cells must first clump together; galectins on their surface membranes are thought to be responsible for much of this metastatic potential. Galactose-rich MCP has a binding affinity for galectins on the surface of cancer cells, resulting in an inhibition, or blocking, of cancer cell aggregation, adhesion, and metastasis. MCP acts as a ligand for galectin-3, which plays a major role in tumor formation and progression. It has been recently shown by using a combination of fluorescence microscopy, flow cytometry, and atomic force microscopy, for the first time, specific binding of a pectin galactan to the recombinant form of human galectin-3. MCP is thought to render galectin-3 incapable of binding its receptors that would result in angiogenesis. Moreover, MCP also shows antimetastatic effects on cancer cells in vitro or in vivo. The first published human clinical trial to use MCP demonstrated its effect on PSA doubling time. A more recent clinical trial that used MCP showed a significant improvement of quality of life and stabilization of disease for patients with advanced solid tumors. One patient in the latter study, who had advanced and hormonal resistant prostate cancer, had a 50% decrease in PSA with significant improvement in his quality of life after taking 15 g of MCP per day. Most of the patients had improvements in their life quality as reflected in the Karnofsky scores.

Apart from therapeutic roles against prostate cancer, MCP has an immunomodulation effect. It can also remove toxic metals from the body. It is also effective against various types of cancer, such as melanoma, colon, and breast cancer. The primary purpose of this study was to confirm the anticancer efficacy of MCP, using the MCPs included in previous cancer clinical trials. MCP is not a carefully defined term as a dietary supplement because there are multiple MCPs on the market that are not well characterized molecularly, and are not the same molecular weight range or low esterification as the MCP marketed under the PectaSol name. Of note, the MCPs studied here are the only MCPs that have been used in human clinical trials. MCP structure to single carbohydrate units. The lower molecular weight PectaSol-C MCP may have better bioavailability than higher molecular weight PectaSol MCP in vivo. In this study, we examined the anticancer effects of PectaSol-C MCP and PectaSol MCP on androgen-dependent and -independent prostate cancer cells. This study was performed to demonstrate and establish the efficacy of the new preparation of PectaSol-C MCP before further chemopreventive and chemotherapeutic analysis in vivo.

**Materials and Methods**

**Reagents**

PectaSol MCP and PectaSol-C MCP (EcoNugenics, Santa Rosa, CA) were added to medium immediately before treatment. Penicillin, streptomycin, RPMI 1640 medium, D-MEM medium, and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA). Anti-pERK1/2, total ERK1/2, Bim, Caspase-3 antibodies were from Cell Signaling (Danvers, MA).

**Cell Culture**

LNCaP and PC3 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS and 1% antibiotic antimycotic (Invitrogen, Carlsbad, CA). Two mouse prostate cancer cell lines, CASP2.1 and CASP1.1 were derived from the tumors of Nkx3.1;Pt;P27 compound mutant mice. Noncancerous benign prostate hyperplasia BPH-1 cell line is kindly provided by Dr Simon W. Hayward. These cells were kept in our laboratory. All the cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

**Cell Viability Assay**

Cells were seeded at 4 × 10³ cells/well in 96-well plates overnight before treatment with MCPs. MTT solution (10 μL; 5 mg/mL in phosphate-buffered saline) was added to each well of the plates and incubated for 3 hours at 37°C. MTT lysis buffer was then added to dissolve the formazan. The optical density was measured at 570 nm using a μQuant Microplate Spectrophotometer (Biotek, Winooski, VT). The percentage of viable cells was calculated as the relative optical density compared to the control.

**Western Blotting Assay**

Cells were lyzed in RIPA buffer containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and phosphatase inhibitor (Sigma, St Louis, MO). Cell lysates (20 μg) were resolved on sodium dodecyl...
sulfate–polyacrylamide gel electrophoresis, and transferred onto a PVDF membrane. After blotting in 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST), the membranes were incubated with primary antibodies at 1:1000 dilutions in TBST overnight at 4°C. The blots were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour, and finally detected by ECL reagent (GE Healthcare, Buckinghamshire, UK).

**Results**

**Growth Inhibitory Effects of PectaSol MCP and PectaSol-C MCP on Prostate Cancer Cells**

We examined the anticancer effects of the MCPs in vitro, with the long-term goal for preclinical study in future. Because we will use *nkx3.1; pten* compound mutant mice for future preclinical study, we chose 2 mouse prostate cancer cell lines, CASP1.1 and CASP2.1, with the losses of *nkx3.1* and *pten* alleles.26 Meanwhile, we also included 2 human prostate cancer cell lines (LNCaP and PC3), as well as 1 noncancerous benign prostate hyperplasia (BPH-1) cells for cytotoxicity study.

At first, we treated androgen-independent CASP1.1 and androgen-dependent CASP2.1 prostate cancer cell lines with the MCPs. Figure 1 shows that treatment with 1.0% of both MCPs for 48 hours induced changes in cellular morphologies (such as cell shrinkage and round-up) in CASP1.1 cells. CASP2.1 cells also turn to be rounded up under both of the treatments; however, the percentage is less than that in CASP1.1. The morphologic changes were dependent on dosage of MCPs. To investigate the cytotoxic effects of MCPs, we performed an MTT assay on 4 different prostate cancer cell lines. Two of them are androgen dependent (CASP2.1 and LNCaP) and the other 2 are androgen independent (CASP1.1 and PC3). The results in Figure 2 indicate that both MCPs can suppress cell proliferation in a dose-dependent manner. Consistently, the data from MTT assay demonstrated that 1.0% of PectaSol MCP exerts cytotoxicity on CASP1.1 cells (46.1% ± 1.7%) more effectively than on CASP2.1 cells (15.4% ± 0.8%); whereas cytotoxicity of
following 1.0% PectaSol-C MCP treatment was greater in CASP1.1 cells (49.0% ± 1.3%) than in CASP2.1 cells (23.0% ± 2.6%). Similarly, both PectaSol and PectaSol-C showed cytotoxic effects on 2 human prostate cancer cells, LNCaP (48.0% ± 2.1%, 52.2% ± 1.8%) and PC3 cells (54.4% ± 0.3%, 48.2% ± 2.9%), respectively. Although 1.0% of PectaSol and 1.0% of PectaSol-C MCPs also showed cytotoxicity on human BPH-1 cells (27.4% ± 1.6% and 26.8% ± 2.6%), respectively, the cytotoxic effects are significantly lower (all \( P \) value <.05) than human prostate cancer cells (LNCaP and PC3). These data indicate that both MCPs have anticancer properties with PectaSol-C MCP showing increased cytotoxicity in most of the cell lines.

**Figure 2.** Effects of PectaSol and PectaSol-C on cell viability on 2 androgen-dependent prostate cancer cell lines (LNCaP and CASP 2.1) and 2 androgen-independent prostate cancer cell lines (PC3 and CASP 1.1), as well as 1 noncancerous human benign prostate hyperplasia BPH-1 cells.

The cells were exposed to modified citrus pectins at the indicated dosages. The cytotoxicity was determined by MTT assay. Vehicle-treated cells were arbitrarily set as 0% of cytotoxicity. Error bars and all data were expressed as mean ± standard error in triplicate. *\( P < .05\), compared with the no-treatment group.

**Cytotoxic Effects of PectaSol MCP and PectaSol-C MCP on PC3 and CASP1.1 Prostate Cancer Cells by Suppression of MAPK signaling and Induction of the Cleavage of Caspase-3**

To confirm the cytotoxicity of the MCPs on prostate cancer cells, we performed Western blotting assay on PC3 cells, as well as CASP1.1 cells. It revealed that both MCPs can suppress mitogen-activated protein kinase (MAPK) signaling through reducing phosphorylation forms of ERK1/2 (Figure 3). Bim, a pro-apoptotic member of the Bcl-2 protein family member, was reported to be targeted by ERK1/2 through phosphorylation and eventually leading to...
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degradation.28,29 We observed an increase of Bim protein levels in both cell lines, after treatment of MCPs, with the inactivation of ERK1/2. Consistently, we also detected the cleavage of pro-Caspase-3 after treatment of MCPs in both PC3 and CASP1.1 cell lines. These results indicate that both MCPs induce cytotoxic effects through the inhibition of prosurvival MAPK signaling and the induction of apoptosis by the activation of Caspase-3.

Discussion

In this study, we showed that both MCPs can induce cytotoxicity in both androgen-dependent and -independent prostate cancer cells in vitro. The anticancer effects of both MCPs are very similar in most of cancer cells we tested. MAPK signal pathway was impaired by the treatment of MCPs. The lower average molecular weight and monogalacturonic acid content of PectaSol-C MCP may be important for the absorbance and distribution in the body.

MCP has shown to have positive impact clinical benefit for patients with advanced solid tumors.21 For prostate cancer patients, MCP may lengthen the PSA doubling time in those with recurrent prostate cancer.11 Our studies show that both PectaSol MCP and PectaSol-C MCP showed cytotoxicity on human and mouse prostate cancer cells. Although we observed some cytotoxicity in the BPH-1 cell line, there appears to be selectivity toward the cancerous cell lines. For mechanistic study, both MCPs can induce apoptosis in androgen-independent PC3 and CASP1.1 prostate cancer cells. The cleavage of Caspase-3 suggests that the induction of apoptosis by the MCPs may be caspase-3 dependent. Moreover, these MCPs can strongly suppress the MAPK signaling pathway, which is related to prostate cancer cell proliferation and survival,30 manifested by the reduction of phosphorylation of ERK1/2. Of note, the reduction of ERK1/2 by MCPs correlates with the increase of Bim protein levels in both cell lines, indicating that the effect is not cell line dependent. Bim is a BH3-only protein, promoting apoptosis through binding to prosurvival Bcl-2 proteins, thereby releasing pro-apoptotic Bax or Bak proteins and eventually leading to apoptosis.31 ERK1/2 has recently shown to phosphorylate Bim to facilitate its proteasome-dependent degradation.28,29 Therefore, these MCPs can inhibit cell growth through the inhibition of the prosurvival MAPK pathway to induce apoptosis.

MCP was shown to inhibit cell adhesion of breast cancer cells to human umbilical vein endothelial cells (HUVECs), through binding to and suppressing the activity of galectin-3.10 A notable feature of galectin-3 is its implication in neoplastic transformation and cancer progression.14 Anti-galectin-3 antibody inhibited adhesion of cancer cells and liver metastasis by adenocarcinoma cell lines.32 These studies suggest the potential for carbohydrate-mediated cancer therapy.

This study shows that PectaSol-C, a promising MCP with average lower molecular weight, has similar anticancer effects to previous MCP with average slightly higher molecular weight. Although this study is not conclusive, the data shown here strongly suggested that PectaSol-C may be a promising anticancer agent for prostate cancer cells and further in vivo study is warranted.

Figure 3. Involvement of MAPK signal pathway and Caspase-3 in PC3 (A) and CASP1.1 (B) prostate cancer cell growth inhibition and apoptosis by PectaSol and PectaSol-C at indicated concentrations.

Western blotting analysis of phosphorylated ERK1/2 at threonine 202 and tyrosine 204, Bim and Caspase-3 in PC3 and CASP1.1 cells. Cells were treated with modified citrus pectins at the indicated dose. An amount of 20 μg proteins were loaded for the assay. Total ERK1/2 was used as a loading control. FL, full length; cCaspase-3, cleaved Caspase-3.
In summary, our results demonstrated that both MCPs can efficiently reduce cell viability in vitro, independent of their androgen dependency. These MCPs can induce cell growth inhibition and apoptosis partially through the inhibition of MAPK signal pathway and activation of Caspase-3. These data support the role of these specific dietary carbohydrate compounds as chemopreventive and/or therapeutic agents, and further clinical and basic science data is warranted in this area.

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