Research Paper

Coix Seed Extract, a Commonly Used Treatment for Cancer in China, Inhibits NFκB and Protein Kinase C Signaling

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INTRODUCTION

The Coix lachryma-jobi seed has long been used in traditional Chinese medicine for treatment of various ailments, particularly cancer. The Zhejiang Province of China, where this seed is routinely used as a food source, reportedly has a relatively low cancer rate that cannot be explained by other risk factors. In recent years, a standardized, pharmaceutical-grade emulsion of Coix seed oil was approved by the Chinese Ministry of Public Health and, based on clinical evidence for anti-neoplastic activity,¹⁻⁴ this preparation has been used in to treat over 500,000 patients in China for treatment of various common types of cancer, including lung cancer, breast cancer, and liver cancer.⁵ In 2005, this Coix extract emulsion was approved for treatment of lung cancer in Russia, and with a recent completion of a phase I clinical trial, this preparation is also being evaluated for cancer therapy in the United States.

Although this Coix extract preparation is the most widely used cancer treatment in the most populous country in the world, there is scant data available regarding potential mechanisms of activity. To verify anti-neoplastic activity of this extract, we first treated nude mice with xenografts of human MDA-MB-231 breast cancer cells with daily intraperitoneal injections of the emulsion. Then, to investigate potential mechanisms, we initially examined effects of Coix extract on gene expression patterns of the MDA-MB-231 cells using oligonucleotide microarrays. Noting a robust downregulation of expression of genes related to cancer invasion and metastasis in cells treated with Coix extract, we extended our studies to pathways that regulate expression of these genes.

MATERIAL AND METHODS

Cell culture and xenografts. MDA-MB-231 human breast cancer cell line and H460 human lung cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown in complete medium containing RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma),

ABSTRACT

A pharmaceutical grade extract of Coix lachryma-jobi seeds is currently the most commonly used treatment for cancer in China. Although clinical data support the use of this preparation of a Traditional Chinese Medicine for cancer treatment, biological basis for the activity of this preparation has not been previously established. To address this issue, we first evaluated the anti-neoplastic activity of a Coix extract emulsion in xenografts of MDA-MB-231 breast cancer cells and found that the extract significantly inhibits growth of MDA-MB-231 xenografts in athymic nude mice. Using oligonucleotide microarrays, we determined that Coix seed extract also significantly affects gene expression in these cells, including downregulation of genes (such as COX-2 and matrixmetalloproteinases) that are considered to be important in neoplasia. The specific gene expression changes noted after Coix seed extract treatment are characteristic of inhibition of NFκB-dependent transcription, leading us to evaluate how the treatment affects that pathway. An NFκB-dependent reporter assay demonstrated dose-dependent inhibition of NFκB signaling by treatment of cultures with the extract, and immunofluorescent microscopy found that these effects are associated with reduced translocation of the Rel-A/p65 subunit of NFκB to the nucleus. Coix extract also inhibits activity of protein kinase C, a major mediator of signal transduction and activator of NFκB. Thus, this Traditional Chinese Medicine-based cancer treatment affects cellular pathways of recognized importance in neoplasia.

KEY WORDS

Coix extract, Traditional Chinese Medicine, NFκB, COX-2

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Invasion assays were performed using 5 replicates. Fluorescent signal intensities were measured using a hybridized to two different membranes for "dye-swap" technical sample was labeled with two different fluors (Cy3 and Cy5) and PMA (phorbol myristate acetate, EMD Biosciences; San Diego, CA). After transfer to nitrocellulose membrane (Millipore, Bedford, Mass.) and blocking with 5% non-fat dry milk in TBS-T (20 mM Tris-HCl, 0.5 M NaCl, 0.2% Tween20), specific peptides were measured by incubating with primary antibodies followed by washes, incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and ECL detection (Amersham Biosciences, UK). Actin protein levels were measured using same methods as control for constant loading and transfer.

Cell transfections/reporter assays. MDA-MB-231 cells (at 70% confluence) were co-transfected with the NFκB-Luc construct (Clontech; Palo Alto, CA), which links a firefly luciferase reporter to an NFκB response element and a renilla luciferase construct (Promega, Madison, WI). To prepare DNA for transfection, 6 μl of LipofectAMINE (Invitrogen, Carlsbad, CA) and 2 μg DNA were diluted with 0.2 ml of OPTI-MEM (Invitrogen) according to manufacturer's instructions. After 24 hours culture, the transfected cells were cultured with or without increasing doses of Coix extract. After an additional 24 hours, cell lysates were prepared and luminescence was detected using a Dual-Luciferase Reporter Assay System (Promega; Madison, WI) with a Victor2 fluorometer (PerkinElmer, Life Sciences, MA). Data is reported as the average of three independent experiments.

Gelatin substrate gel zymography. To determine the effect of the Coix extract on MMP-9 activity, cells were treated with various concentrations of PMA and coix extract with 20 nM PMA and MMP-9 expression was evaluated by zymography. Zymography was performed using previously described methods. Supernatants were collected from cell cultures (80% confluent) after 24 hr treatments with designated concentrations of Coix extract and PMA, and proteins were separated by electrophoresis in 10% polyacrylamide gels that were copolymerized with 1 mg/ml of gelatin. After electrophoresis, the gels were washed several times in 2.5% Triton X-100 for 1 h at room temperature to remove the SDS, then incubated for 24–48 h at 37°C in buffer containing 5 mM CaCl₂ and 1 μM ZnCl₂. The gels were then stained with Coomassie blue (0.25%) for 30 min, followed by 1 hr wash in a solution of acetic acid and methanol. The proteolytic activity was evidenced as clear bands (zones of gelatin degradation) against the blue background of stained gelatin.

In vitro invasion assay. Invasion assays were performed using modified Boyden chambers with polycarbonate nucleopore membrane (Corning, Corning, NY, USA) by plating 4 x 10⁴ cells/chamber. Precoated filters (6.5 mM in diameter, 8 μM pore-size, Matrigel 100 μg/cm²) were rehydrated with 250 μl of medium, and 5 x 10⁴ cells in 200 μl medium with or without Coix extract in the presence of 20 nM PMA were seeded into the upper part of each chamber. Following incubation for 24 h at 37°C, non-migratory cells on the upper surface of the filter were wiped with a cotton swab, and migrated cells on the lower surface of the filter were fixed and stained with 0.125% Comassie Blue in a methanol: acetic acid: 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μg/ml streptomycin in humidified air with 5% CO₂ at 37°C. Cells were detached with 0.25% trypsin-0.02% EDTA and 2–6 x 10⁵ exponentially growing cells were plated into 100-mm tissue culture dishes or 0.5–1 x 10⁶ into 6-well plates in complete medium. After culture overnight in complete medium, cells were treated with drugs at different concentrations as indicated in each figure legend in complete medium at 37°C for the indicated time intervals. Xenografts were established by injecting 1 x 10⁶ cells into the subcutaneous fat pads of female athymic nude mice (NCl, Frederick, MD). Two days after implantation, animals were randomized to treatment or control groups (ten mice per group), and then treated daily with 50 μg of Coix seed oil extract (500 μl emulsion) or RPMI culture medium. Tumor volume was measured weekly with calipers, and experiments were terminated when morbidity was observed in control animals.

Cox seed oil emulsion. The Coix seed oil emulsion was prepared by the Zhejiang Kanglai Pharmaceutical Co., using seeds harvested from specified fields in the Zhejiang Province of China. No chemical fertilizers or pesticides were used in raising the crops, and plantings and harvestings were conducted at pre-determined times. The water-based emulsion was prepared to contain 10 g Coix seed oil per 100 ml of emulsion, with soybean phospholipid (1.5 g) and glycerin (2.5 g) added as emulsifiers. All batches were analyzed by high performance liquid chromatography (HPLC) using an Agilent 1100 HPLC with quaternary pump and an Alltech 500 ELSD detector. The temperature of drift tube was 70°C, with air at a flow rate of 1.2 L/minute used as carrier gas in a Zorbax Extend C18 column (4.6-mm internal diameter and 250-mm length (Agilent Technologies, Santa Clara, CA) at 35°C, acetoni trile-dichlormethane (65:35) as mobile phase, at a 0.5 ml/min flow rate. Integration on each test result was conducted with the slope rate of 60 in the Agilent HP ChemStations system. Results of HPLC analysis for lots used in this study are shown in Figure 1.

Microarray hybridization signal detection and data analysis. Expression levels of genes in cultured cells treated with Coix extract and PMA (phorbol myristate acetate, EMD Biosciences; San Diego, CA), alone or in combination, were compared to control cultures by oligonucleotide microarray analysis (Agilent Technologies, Santa Clara, CA), using manufacturer-recommended protocols. Each sample was labeled with two different fluors (Cy3 and Cy5) and hybridized to two different membranes for "dye-swap" technical replicates. Fluorescent signal intensities were measured using a GenePix 4000 A fluorescent scanner (Axon Instruments, Sunnyvale, CA), and signal intensity data were extracted using GenePix 4.0 microarray analysis software.

Western blot analysis. Cellular lysates were prepared and proteins separated (typically 50 μg of total protein) by electrophoresis using previously described methods. For cell fractionation, we used a ProteoExtract® Subcellular Proteome Extraction Kit with differential solubility of subcellular compartments (EMD Biosciences; San Diego, CA). After transfer to nitrocellulose membrane (Millipore, Bedford, Mass.) and blocking with 5% non-fat dry milk in TBS-T (20 mM Tris-HCl, 0.5 M NaCl, 0.2% Tween20), specific peptides were measured by incubating with primary antibodies followed by washes, incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and ECL detection (Amersham Biosciences, UK). Actin protein levels were measured using same methods as control for constant loading and transfer.

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Protein kinase C activity was measured as phosphorylation of a synthetic peptide substrate (PepTag C1, Promega). Two micrograms of peptide were incubated with purified enzyme or cell extract, according to the manufacturer's instructions. Reactions were stopped by heating to 95°C for ten minutes, and reaction products were separated on a 0.8% agarose gel at 100V for 30 minutes. Phosphorylated and unphosphorylated substrates were differentiated by differential migration on the gel.

RESULTS

Coix seed oil extract inhibits growth of MDA-MB-231 human breast cancer xenografts. In our initial experiments, we determined whether the Coix extract preparation could affect growth of human cancer xenografts in athymic mice. For the MDA-MB-231 breast cancer cell line xenografts, we observed significant reduction in tumor growth in the animals treated with 50 µg of Coix seed extract by intraperitoneal injection (Fig. 2). A reduced tumor growth was also observed in Coix extract-treated mice bearing xenografts of the H460 human lung cancer cell line, but the difference between treatment and control groups did not meet the level of statistical significance for this experiment (results not shown). Post-mortem examination of all animals revealed no organ-specific toxicity in any of the treated animals, and dosing in these experiments was limited only by the volume of the emulsion that could be injected into the peritoneum on a daily basis.

Coix seed oil extract inhibits expression of genes important for cancer growth, invasion and metastasis. To explore potential mechanisms of this inhibition of tumor growth, we first examined effects of Coix extract on gene expression patterns of the MDA-MB-231 cells, using oligonucleotide microarrays. For this experiment, we also examined the effect of Coix extract on gene expression of MDA-MB-231 cells stimulated by PMA, a compound that is commonly used in the experimental setting to activate protein kinase C (PKC) and related signal transduction pathways that are involved in the malignant phenotype. Several important findings were noted in this analysis. First, and not unexpectedly, Coix extract treatment results in modified expression of a number of genes related to lipid transport and metabolism (Fig. 3). More interestingly, we also noted significant changes in expression for a number of genes that are functionally related to cancer cell growth, invasion and metastasis. For example, Coix extract treatment resulted in a significant decrease in the mRNA levels of PTGS2, the gene that encodes COX-2, an enzyme that regulates prostanoid metabolism and has a central role in cancer biology. Coix extract treatment also resulted in decreases in levels of expression for interleukins 6 and 8 (IL-6, IL-8), which have known importance in myeloma and possibly other forms of neoplasia, and significantly attenuated the PMA induced increases in expression for several matrix metalloproteinase genes, which are involved in cancer cell invasion and metastasis.

Because of the recognized roles of COX-2 and MMP-9 in the malignant phenotype, we explored the effects of Coix extract on expression and activity of these genes with additional experiments. First, we verified inhibition of COX-2 expression by Coix extract using immunoblot measurement of the COX-2 protein in treated cells (Fig. 4A). Furthermore, we confirmed that the induction of COX-2 by PMA is significantly attenuated by the Coix extract (Fig. 4B). Immunohistochemistry was also performed on tumor tissue from animals treated with Coix extract, and these studies confirmed that this inhibition of COX-2 expression also occurs in the in vivo setting (Fig. 4C).
MMMP9 is typically secreted from cells, and therefore to confirm effects of Coix extract on expression of this protease, we measured proteolytic digestion of gelatin by supernatants of cultures treated with combinations of Coix extract and PMA. Unstimulated cells have relatively low levels of MMP9 activity, but PMA stimulation results in a rapid increase of activity (Fig. 5A), and Coix extract treatment significantly attenuates this response.

We extended these studies to evaluate ability of Coix extract to affect migration of MDA-MB-231 cells through a basement membrane invasion chamber assay. This assay has been used previously to measure MMP9 activity,12 although other mediators inhibited by Coix extract (including COX-213) may have roles in cell migration and invasion. Our experiments found that Coix extract significantly attenuates the induction of the invasive phenotype induced by PMA in MDA-MB-231 cells as measured by this assay (Fig. 5B and C).

Coix seed oil extract inhibits NFκB signaling. The pattern of gene expression changes induced by Coix extract treatment, as described above, strongly suggests activity affecting the NFκB transcription factor. For example, expression levels of COX-2, IL6, IL8 and various MMPs have all been previous found to be regulated by NFκB in various cells.14,15 We therefore investigated the possible mechanistic role of this pathway in mediating the effects of Coix seed oil emulsion in a series of experiments. First, we used an NFκB-dependent luciferase reporter assay to directly measure effects of the preparation on a gene promoter element that has a consensus NFκB response element. As shown in Figure 6A, activity of this reporter is stimulated by treatment with PMA, and activity in PMA-stimulated cells, as well as in non-stimulated cells, is diminished by Coix seed oil extract in a dose-dependent manner.

The p65/Rel-A subunit of the NFκB transcription factor is regulated by binding to IκBα in the cytoplasm, and activation of the transcription factor (usually by phosphorylation of IκBα and release of p65/Rel-A) is associated with translocation of this transcription factor to the nucleus.16,17 To determine whether Coix extract might affect this process, we examined effects of this preparation on subcellular localization of the p65/Rel-A subunit of NFκB. Using immunofluorescence to detect localization of p65/Rel-A (Fig. 6B), we noted that the relative levels of nuclear staining for this protein in MDA-MB-231 cells increase significantly after stimulation by PMA, consistent with activation of the transcription factor, and this effect is markedly attenuated by treating cell cultures with Coix seed oil extract, in a dose-dependent manner. Finally, we conducted immunoblot analysis of nuclear and cytoplasmic fractions of cells treated with Coix extract (Fig. 6C), and confirmed the inhibition of p65/Rel-A translocation by treatment.

Coix seed oil extract inhibits protein kinase C activity. Together, our results pointed to protein kinase C (PKC) activity as a possible proximal target of Coix extract activity. For example, PMA is a mimic of diacylglycerol, the canonical stimulant of PKC, and...
Coix extract directly inhibits effects of PMA. Furthermore, PKC affects NFKB, either directly through phosphorylation of IκBα, or through activation of the MEK-ERK pathway via Raf1,18,19 which in turn can lead to phosphorylation of IκBα and nuclear translocation of NFKB (ref. 20). To explore the possible involvement of PKC, we examined Coix extract activity with an in vitro assay that uses a fluorescent PKC substrate peptide as an indicator of kinase activity. Cultures of MDA-MB-231 cells were treated with various combinations of PMA and Coix extract for one hour, and then lysates of these cultures were then incubated with the labeled substrate to determine PKC activity. As shown in Figure 7A, the Coix extract resulted in a rapid and significant decrease in kinase activity in cultures that were not treated with PMA as well as those treated with PMA. We also observed a similar level of inhibition of activity by Coix extract using PKC purified from rat brain (Promega), rather than cell lysate, as a source of PKC (data not shown), suggesting that Coix extract has a direct effect on the kinase activity.

To confirm effects of Coix extract on PKC, we used immunofluorescence staining to examine the effect of Coix extract treatment on PMA-dependant translocation of PKC from cytosol to cell membrane, recognizing that this membrane translocation is closely associated with activation of the kinase.21,22 As demonstrated in Figure 7B, Coix extract treatment results in a significant reduction the translocation of PKCα from cytosol to membrane, in response to PMA stimulation, indicating that this important function of PKC is inhibited by the extract.

**DISCUSSION**

Although not widely known in Western countries, this pharmaceutical-grade preparation of a traditional Chinese medicine is now one of the most widely used treatments for cancer in the world. Various publications in the Chinese medical literature report that Coix extract has shown efficacy in clinical trials for treatment of lung cancer, breast cancer, and hepatocellular carcinoma, with minimal adverse effects. Laboratory studies that have been conducted in China indicate that this extract inhibits cell replication or induces apoptosis in some cancer cell lines. However, the anti-neoplastic activity of this preparation has not been reported previously in the Western scientific literature, and a molecular basis for the activity of this extract has not been previously demonstrated.

Our experiments reveal that Coix extract significantly inhibits the growth of human MDA-MB-231 breast cancer xenografts. Dosing in our experiments was limited only by the volume of emulsion that could be injected into the animals, and thus the full potential efficacy cannot be determined by these experiments. Moreover, this preparation is commonly used in combination with conventional chemotherapy,1-4 and evaluating possible additive or synergistic effects of such combinations will be important for understanding the potential role of this preparation in cancer treatment.

A major goal of our experiments was to determine whether this preparation affects cellular pathways that are important in the cancer phenotype, thus providing scientific plausibility for the activity of the extract as well as clues for potential mechanisms. Our results provide multiple lines of evidence that Coix extract attenuates NFKB signaling, including (1) microarray experiments showing that Coix extract induces expression changes for genes regulated by this transcription factor, (2) demonstration that the extract inhibits activity of an NFKB response element and (3) immunofluorescent microscopy showing that Coix extract inhibits translocation of the Rel-A/p65 subunit to the nucleus. In addition, we determined that PKCα is a specific molecular target for Coix extract, with inhibition of this enzyme seen at doses that are not cytotoxic to cells. This inhibition of PKC could be responsible, at least in part, for the decreased NFKB transcriptional activity18,19 and decreased expression of mediators of the malignant phenotype, such as COX-2. Thus, it is evident that this extract affects major pathways involved in cancer.

NFKB and PKC are both recognized as targets for cancer therapy,23-25 with particular interest in the possible use of NFKB inhibitors to enhance other anti-tumor agents via increased apoptosis.26-27 Interestingly, other natural compounds and traditional medicines, including resveratrol and curcumin also appear to target
Coix extract inhibits NFκB signaling. These pathways, 28,29 Coix extract, however, is unique among natural compounds and traditional medicines with respect to the refined level of processing and standardization, extensive clinical use, and demonstrated efficacy in controlled clinical trials. An improved understanding the biological basis of this preparation will help to refine its use in the clinical setting.

References