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Rosemary extract reduces Akt/mTOR/p70S6K activation and inhibits proliferation and survival of A549 human lung cancer cells

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ABSTRACT

Compounds of plant origin and food components have attracted scientific attention for use as agents for cancer prevention and treatment. Rosemary extract contains polyphenols that were shown to have anti-cancer and other health benefits. The survival pathways of Akt, mammalian target of rapamycin (mTOR) and p70S6K, and the apoptotic protein poly ADP ribose polymerase (PARP) are key modulators of cancer cell growth and survival.

In this study, we examined the effects of rosemary extract on proliferation, survival and apoptosis of human non-small cell lung cancer (NSCLC) cells and its influence on signaling events.

Human NSCLC adenocarcinoma A549 cells were used. Cell proliferation and clonogenic survival were assessed using specific assays. Immunoblotting was used to examine total and phosphorylated levels of Akt, mTOR and p70S6K, and cleavage of PARP.

Rosemary extract dose-dependently inhibited cell proliferation and reduced clonogenic survival of A549 cells, while PARP cleavage, an indicator of apoptosis, was enhanced. Rosemary extract significantly reduced total and phosphorylated/activated Akt, mTOR and p70S6K levels.

In conclusion, rosemary extract inhibited proliferation, blocked clonogenic survival, and enhanced apoptosis of A549 lung cancer cells. These effects were associated with inhibition of Akt and downstream mTOR and p70S6K activity. Our data suggest that rosemary extract may have considerable anti-tumor and chemoprevention properties in lung cancer and deserves further systematic investigation in animal models of lung cancer.

1. Introduction

Lung cancer is responsible for the most cancer related deaths [1]. Importantly, non-small cell lung cancer (NSCLC) accounts for up to 80% of all lung cancer cases [2] and represents the most aggressive form of the disease. The 3 main subtypes of NSCLC include adenocarcinoma, squamous cell carcinoma and large cell carcinoma, with adenocarcinomas representing more than 40% of all NSCLC cases [3]. Unfortunately, fewer than 15% of individuals with NSCLC reach 5-year survival despite the use of aggressive chemo- and radiation therapies [4]. Patients with NSCLC often develop resistance to these cytotoxic therapies [5], highlighting the need for novel anticancer strategies.

Cancer cells are characterized by their ability to evade homeostasis and grow (proliferate) uncontrollably while avoiding programmed cell death (apoptosis) [6]. They often acquire these characteristics through mutations to key signaling molecules which regulate pathways involved in cell proliferation and survival [7]. The phosphatidylinositol 3-kinase (PI3K)-Akt pathway is commonly mutated in cancer leading to enhanced cell proliferation and inhibition of apoptosis [5,8]. Activated PI3K leads to downstream activation of the serine threonine kinase Akt (protein kinase B, PKB) [9,10]. Activated Akt in turn leads to activation of the mammalian target of rapamycin (mTOR) and p70 S6 kinase, resulting in stimulation of protein synthesis, growth and proliferation [9,10]. Activated Akt also phosphorylates Bad, a proapoptotic Bcl-2 family member, causing its degradation resulting in inhibition of apoptosis [9,10]. Akt is a proto-oncogene

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and activated Akt leads to cancer cell proliferation, survival, and resistance to chemo and radio-therapy [5,9–11]. Several studies have shown that Akt is overexpressed in various cancer cell lines and human tumors [9,11] leading to enhanced signaling through this pathway. Increased Akt phosphorylation, a marker of activation of this molecule [10], is seen in many cancers [9,11–13]. On the other hand, targeting/inhibiting the Akt signaling pathway inhibits cancer cell growth and enhances apoptosis [9,11].

While apoptosis is an important mechanism to maintain homeostasis in healthy cells, cancer cells often develop an ability to evade apoptosis and thus, this hallmark characteristic of cancer cells represents an important target for cancer prevention/therapy. Apoptosis usually leads to morphological changes in the cell including nuclear condensation, membrane blebbing, chromatin condensation and DNA fragmentation [14]. The poly ADP ribose polymerase (PARP) family of proteins are enzymes which become activated by being cleaved, leading to either DNA repair or, in the case the cell is not repairable, apoptosis. PARP-1, the most abundant PARP protein *in vivo*, is a DNA nick sensor enzyme which is activated by DNA single or double strand breaks [15]. These DNA strand breaks are induced by cell stress, including reactive oxygen species, reactive nitrogen species and ionizing radiation, among others [16]. Activated PARP-1 catalyzes the cleavage of NAD^+ into nicotinamide and ADP-ribose and uses the latter to synthesize branched polymers that attach to nuclear acceptor proteins. The branched poly(ADP-ribose) polymer may attempt to recruit DNA repair enzymes or, if damage is excessive, induce apoptosis. When apoptosis is induced the polymer is degraded, resulting in depleted NAD^+ in the cell which leads to malfunctioning glycolysis, Krebs cycle, mitochondrial electron transport and eventually to ATP depletion and cell death [17], reviewed in Ref. [18].

Many pharmaceutical and chemotherapeutic agents have been discovered by screening chemicals found in plants. The chemotherapeutics etoposide, isolated from the mandrake plant and Queen Anne's lace, and paclitaxel and docetaxel, isolated from the wood and bark of the Nyssaceae tree, are currently employed in cancer treatment [19]. Many labs, including ours, have shown metformin, a drug derived from the lilac, has anticancer properties [20–22]. In addition, the polyphenol resveratrol, found in high concentrations in wine, has been shown to have anticancer effects *in vitro* [23,24] and *in vivo* [24]. Plants rich in polyphenols have received much attention for their anticancer properties, among other health benefits. The exploration into natural products offers great opportunity to evaluate new chemical classes of anticancer agents as well as novel and potentially relevant mechanisms of action.

Rosemary extract (RE) and some of its polyphenol components including carnosic acid (CA), rosmarinic acid (RA) and carnosol (CN), have been explored and found to exert potent anticancer effects. Studies using colon [25–31], breast [32–34], pancreatic [35], prostate [36] and liver [34,37], cancer cells have shown inhibition of cancer cell proliferation and viability, and induction of apoptosis in response to treatment with RE. A limited number of studies have also examined the effects of RE administration on tumor growth in animals *in vivo*. Inhibitory effects have been documented in models of colon, prostate, and skin cancer, as well as myeloid leukemia [36,38–41]. Taken together, these studies provide evidence of important properties of RE to inhibit tumor growth. Despite this evidence supporting the potential of RE as an anticancer agent, limited data exists regarding its effects in lung cancer and little is known about the signaling mechanisms responsible for its anticancer properties. The purpose of this study was to examine the effects of RE treatment on human lung cancer cell proliferation, survival and apoptosis and highlight the benefit of using the extract to combat cancer cell survival. We hypothesized that RE treatment would inhibit A549 lung cancer cell proliferation and survival, enhance apoptosis, and modulate signaling pathways involved in cell growth and survival.

2. Materials and methods

2.1. Materials

Human A549 NSCLC cells were obtained from American Type Culture Collection (ATCC). Cell culture (RPMI) media, fetal bovine serum (FBS), trypsin, and antibiotic were from GIBCO (Burlington, ON, Canada). Total and phospho-specific antibodies (Akt, mTOR, p70S6K, PARP) were from Cell Signaling Technology *via* New England Biolabs (Mississauga, ON, Canada). The β-actin antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Resveratrol, bovine serum albumin and dimethyl sulfoxide (DMSO) were purchased from Sigma (Oakville, ON).

2.2. Preparation of rosemary extract

Dried rosemary leaves were purchased from Sobeys (Mississauga, ON) and the rosemary extract was prepared as reported previously [42,43]. Briefly, dried rosemary leaves were ground and steeped overnight in dichloromethane: methanol (1:1) followed by filtration the next day. After filtering, the liquid solvent was set aside while the leaves were boiled in methanol for 30 min. The liquid solvent obtained after boiling was combined with the filtered liquid solvent. The combined solvent was removed from the final extract by rotary evaporation and the green powder was collected and stored at −20 °C, protected from light.

2.3. Cell culture and treatment

A549 cells were grown in RPMI media supplemented with 10% (v/v) FBS, and 1% (v/v) antibiotic-antimycotic solution in a humidified atmosphere of 5% CO_2 at 37 °C. A stock solution of 100 mg/mL RE, dissolved in DMSO was prepared and stored at −20 °C, protected from light until use. Further dilutions were made in 10% FBS-containing RPMI media immediately before cell treatment began. The final concentration and the time of incubation with RE, resveratrol or DMSO are indicated in each figure.

2.4. Cell proliferation assay

The crystal violet cell proliferation assay was performed as described previously [44]. Briefly, A549 cells were seeded (1000 cells/well) in triplicate, incubated with the indicated concentration of RE, resveratrol, or DMSO for 72 h, fixed and stained with crystal violet dye. The following day dye was solubilized and absorbance was read at 570 nm using a KC4 plate reader (Bio-Tek). Results are expressed as percent of untreated control.

2.5. Clonogenic assay

Clonogenic assays were performed as described previously [44]. Briefly, A549 cells were seeded in triplicate in 96 well plates (800 cells/well), allowed to adhere overnight and incubated with media containing the indicated concentrations of rosemary extract for 7 days. Cells were then fixed and stained with 0.05% methylene blue and colonies (>50 cells) were counted. Results are expressed as the surviving fraction compared to untreated control.

2.6. Immunoblotting

After treatment cell lysates were prepared, protein content was measured using the Biorad protein assay and samples (20 μg) were