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Antrodia cinnamomea fruiting bodies extract suppresses the invasive potential of human liver cancer cell line PLC/PRF/5 through inhibition of nuclear factor κB pathway ☆

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Abstract

In this study, we first report the anti-invasive effect of ethylacetate extract from *Antrodia cinnamomea* (EAC) fruiting bodies in the human liver cancer cell line PLC/PRF/5. Treatment with EAC decreased the cancer invasion of PLC/PRF/5 cells in a dose-dependent manner. This effect was strongly associated with a concomitant decrease in either the level or activity of VEGF, MMP-2, MMP-9 and MT1-MMP, and an increase in the expression of TIMP-1 and TIMP-2. EAC inhibited constitutively activated and inducible NF-κB in both its DNA-binding activity and transcriptional activity. Furthermore, EAC also inhibited the TNF-α-activated NF-κB-dependent reporter gene expression of MMP-9 and VEGF, and the invasion of cancer cells. EAC also exhibited an inhibitory effect on angiogenesis in a Matrigel Plug Angiogenesis Assay. Further investigation revealed that EAC's inhibition of cancer cell growth and invasion was also evident in a nude mice model. Our results indicate that EAC inhibits the activation of NF-κB, and may provide a molecular basis for drug development using EAC as an anti-invasive agent in the prevention and treatment of cancer.

Keywords: *Antrodia cinnamomea*; Invasion; Liver cancer; MMPs; NF-κB

1. Introduction

Tumor invasiveness and metastasis are characteristics of highly malignant cancers with poor clinical outcome (Qin and Tang, 2004; Christofori, 2006). Tumor invasion is a perplexing cascade process involving a finely tuned interaction between cancer cells and various regulated factors (Christofori, 2006). As cancer cells become invasive and metastatic, they alter the affinity of extracellular matrix (ECM), such as basement membrane. Excess breakdown of ECM is one of the hallmarks of tumor invasion and metastasis (Christofori, 2006; Huang et al., 2002; Hofmann et al., 2005). MMPs are members of zinc-dependent endopeptidases family which is associated with the degradation of ECM during tissue remodeling. Among all MMPs, MMP-2 and MMP-9 (gelatinase A and B, respectively) have been implicated as important factors in facilitating invasion and metastases in liver cancer (Hofmann et al.,

☆ The generic name of *Antrodia camphorata* is currently corrected by Chang and Chou (Chang and Chou, 2004).

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2005; Hou et al., 2001). MMPs have a unique capability to degrade type IV collagen, which is a major component of the basement membrane. Overexpression of MMP-2/-9 has been associated with increased metastatic potential in many tumor cells (Hofmann et al., 2005). In contrast to MMP-2 and MMP-9, which are secreted and as soluble enzymes, membrane-type MMPs (MT-MMPs) contain either an additional transmembrane domain (MT-1-, MT-2-, MT-3-, and MT-5-MMP) or a glycosylphosphatidyl-inositol anchor (MT-4-, and MT-6-MMP), resulting in cell surface localization. MT-1-MMP degrades a number of ECM proteins, stimulates angiogenesis, promotes tumor invasion and growth, and is also involved in activation of proMMP-2 (Hofmann et al., 2005; Zucker et al., 2000; Ko et al., 2005). Indeed, the activity of MMPs is regulated by a series of naturally occurring tissue inhibitors of matrix metalloproteinases (TIMPs). At least four different structurally related members (TIMP-1 to TIMP-4) have been identified (Ikenaka et al., 2003). The TIMPs bind either to proMMPs or active MMPs, thereby inhibiting the autocatalytic activation of latent MMP enzymes and the proteolytic capacity of active proteinases (Chirco et al., 2006). Furthermore, vascular endothelial growth factor (VEGF), the most well-characterized angiogenic factor, is known to play an important role in tumor-associated microvascular invasion (Huang et al., 2005).

The transcription factor nuclear factor κB (NF-κB) regulates the expression of a wide variety of genes involved in cellular events such as inflammation, immune response, proliferation, apoptosis, and cancer invasion (Dixit and Mak, 2002; Herr and Debatin, 2001; Woodworth et al., 2005). NF-κB has been reported to modulate the expression of several genes whose products are associated with tumor development (Aggarwal, 2004; Karin et al., 2002). These include antiapoptotic genes (i.e., c-FLIP, Bfl-1, Bcl-2, Bcl-X_L, and XIAP), cancer invasiveness genes (i.e., matrix metalloproteinases (MMP)-9, urokinase, VEGF and COX-2) and cell cycle-related genes (i.e., c-Myc and cyclin D1) (Denoyelle et al., 2001; Shishodia et al., 2005; Srivastava and Singh, 2004). NF-κB can also decrease the induction of apoptosis mediated by genotoxic chemotherapeutic agents and ionizing radiation. Cancer cells in which NF-κB is constitutively active are highly resistant to anticancer agents or ionizing radiation, and inhibition of NF-κB activity in these cells greatly decreases their invasiveness (Dixit and Mak, 2002; Herr and Debatin, 2001; Munshi et al., 2004).

In our earlier report, we showed that EAC inhibits liver cancer cells by inducing apoptosis in Hep G2, PLC/PRF/5, and Hep 3B (Kuo et al., 2006; Hsu et al., 2005b). EAC induces apoptosis of Hep 3B cells through calcium and calpain-dependent pathways, whereas it decreases NF-κB cell survival signaling in Hep G2 and PLC/PRF/5 cells (Kuo et al., 2006; Hsu et al., 2005b). Therefore, in this study we investigated whether EAC may lead to the down-regulation of invasion-related factor through NF-κB inhibition, thereby inhibiting invasion of human liver cancer cells. Our results suggest that EAC causes a decrease in the expression of MMP-2, MMP-9, MT-1-MMP, and VEGF, resulting in the inhibition of human liver cancer cell invasion.

2. Materials and methods

2.1. Cell invasion assay

BD BioCoat tumor invasion system (BD Biosciences) was used to assess invasion by PLC/PRF/5 cells. Briefly, PLC/PRF/5 cells (5×10^4) with serum-free medium were seeded into the upper chamber of the system. Bottom wells in the system were filled with 20%FBS or TNF-α (100 ng/ml). After 24 h of incubation, the cells in the upper chamber were removed, and the cells which had invaded through the Matrigel matrix membrane were stained with 4 μg/mL Calcein AM in Hanks buffered saline at 37 °C for 1 h. Then, fluorescence of the invaded cells was read in a fluorescence plate reader at excitation/emission wavelengths of 485/530 nm.

2.2. Electrophoretic mobility shift assay (EMSA)

The nuclear extract was prepared using Nuclear Extract kit (Active Motif, Carlsbad, CA) according to the manufacturer's specifications. EMSA was performed by using lightshift chemiluminescent EMSA kit (Pierce, Rockford, IL, USA) following the manufacturer's protocol. Five micrograms nuclear protein, 50 fmole 3′-biotin labeled DNA probes, and double-stranded NF-κB oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′ and 3′-TCAACTCCCCTGAAAGGGTCCG-5′) were mixed in the binding reagent containing 1× binding buffer (100 mM Tris, 500 mM KCl, 10 mM dithiothreitol), (pH 7.5), 2.5% glycerol, 5 mM $MgCl_2$, 50 ng/mL poly (dI–dC), and 0.05% NP-40. After incubation for 30 min, the reaction mixture was subjected to gel electrophoresis on 8% native polyacrylamide gel and transferred to a nylon membrane. The biotin end-labeled DNA was detected using streptavidin-horseradish peroxidase conjugate and chemiluminescence blotting detection system.

2.3. NF-κB reporter assay

NF-κB-dependent reporter plasmid (NF-κB-SEAP), designed to monitor the transcription factor binding activity of NF-κB through the use of a SEAP assay, was purchased from BD Biosciences (San Jose, CA). PLC/PRF/5 cells were transiently co-transfected with NF-κB-SEAP and M1-β-Gal plasmid (Roche Diagnostics, GmbH) using Lipofectamine 2000 reagent. SEAP and galactosidase expression was determined with the SEAP assay system and β-galactosidase assay system respectively (Roche, Germany), according to the reference protocol. SEAP expression was normalized to β-galactosidase expression to control for transfection efficiency. Relative SEAP activity was determined to reflect the transcriptional activity of NF-κB, and expressed as fold increases relative to the activity of untreated controls.

2.4. MMP-2, MMP-9, and VEGF assay

The culture medium of the PLC/PRF-5 cells grown in six-well plates was collected. After collection, the medium was centrifuged at 800*g* for 3 min at 4 °C to remove cell debris. The supernatant was immediately assayed using commercially available MMP-2, MMP-9, and VEGF ELISA kits (R&D Systems, Inc., Minneapolis, MN).

2.5. Gelatin zymography

PLC/PRF/5 cells were treated with 30 μg/ml of EAC in serum-free DMEM medium for 24 h. The conditioned media were collected and analyzed for MMPs using gelatin zymography. MMPs were separated by