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Baicalin suppresses lung carcinoma and lung metastasis by SOD mimic and HIF-1α inhibition

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ABSTRACT

The dose-related toxicity of anticancer drugs in chemotherapy of clinical carcinoma is the major obstacle to prolonged survival, we want to investigate selective therapeutic efficacy of baicalin on lung carcinoma and explain the basis underlying this phenomenon. In vitro, baicalin inhibited cell proliferation of human lung carcinoma A549 and mouse lewis lung cancer (LLC) in a dose- and time-dependent manner. The inhibitory activity of baicalin against cancer cells was promoted by superoxide dismutase (SOD) addition or hypoxia-inducible factor-1alpha (HIF-1α) knockdown and was reduced by SOD knockdown but not hypoxia. In vivo, baicalin suppressed tumor growth and prolonged survival in C57BL/6 mice bearing LLC tumor and nude mice bearing A549 carcinoma without systemic toxicity. Further studies showed that baicalin inhibited HIF-1α and enhanced SOD activity without affecting catalase and glutathione-S-transferase (GST) in cancer cells. In addition, baicalin also exhibited a superoxide anion scavenging activity. In conclusion, baicalin could selectively suppress lung carcinoma and lung metastasis by SOD mimic and HIF-1α inhibition.
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1. Introduction

Lung cancer is the first leading cause of cancer death in the United States (Minna et al., 2002). Unfortunately, despite efforts to improve its prognosis, the overall survival of patients with lung cancer is still unsatisfactory (Gautam and Koshkina, 2003). The high mortality rate is mostly due to the high rate of metastasis and severe resistance to both chemotherapy and radiation (Lebedeva et al., 2007). However, therapeutic selectivity of chemical drugs is also the major obstacle to prolonged survival (Workman, 2004). Therefore, development of novel compounds that selectively eliminate tumor cells with minimal toxicity to normal tissues is an important and challenging task, and understanding of the biological differences between normal and cancer cells is essential to achieve this goal.

The development of intratumoral hypoxia and low glucose levels within the tumor cells is a hallmark of rapidly growing solid tumors (Chan et al., 2007). Adaptation of tumor cells to a hypoxic environment results in a resistance to conventional chemotherapy and radiotherapy, and a poor treatment outcome (Chaudary and Hill, 2007). Hypoxia-inducible factor 1 (HIF-1) is the central mediator of cellular responses to hypoxia and plays a central role in tumor progression and angiogenesis in vivo (Gordan and Simon, 2007). In tumor xenograft models, decreased HIF-1 activity is usually associated with a slower growing and less angiogenic tumor phenotype (Melillo, 2006). Histologic analyses have shown that hypoxia-inducible factor-1alpha (HIF-1α) is overexpressed in many human cancers (Zhong et al., 1999). It is therefore rational to target HIF-1 activity for antitumor intervention.

Another change in cancer cells is the increase in reactive oxygen species generation that plays an important role in maintaining cancer phenotype due to their stimulating effects on cell growth and proliferation (Pelicano et al., 2004). Because of the pro-oxidant status of the cancer cells, they seem to be more susceptible than normal cells to treatment with agents that cause oxidative stress (Laurent et al., 2005). Superoxide dismutase (SOD) can affect tumor cell proliferation via their effects on peroxide levels, overexpression of SOD in human cancer cell lines increases H_2O_2 production (Zhang et al., 2002). In addition, overexpression of SOD in human cancer cell lines also suppresses hypoxic accumulation of HIF-1α protein under hypoxic conditions (Kaewpila et al., 2008). Therefore, SOD mimic should also reduce tumor growth with minimal affecting normal tissues having higher endogenous antioxidant protein levels. We hypothesized that a single molecule with SOD mimic and HIF-1α inhibitory properties could be beneficial through its ability to enhance the antitumor activity while minimizing systemic toxicity. Baicalin (7-glucuronic acid, 5,6-dihydroxy-flavone) is a flavonoid present in many traditional Chinese medicines. A number of studies show that baicalin has

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anticancer effects and protects against a variety of tissue and organ injuries (Min et al., 2008). In recent studies, baicalin potentiates SOD activity and reduces HIF-1α stabilization (Liu et al., 2008; Cho et al., 2008). In this study, we evaluated the selective therapeutic efficacy of baicalin on lung carcinoma both in vitro and in vivo.

2. Materials and methods

2.1. Materials

Baicalin (high performance liquid chromatographic content > 98%) was from Shanxi Huike Botanical Development Co. (China). RPMI1640 medium was from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS), penicillin, and streptomycin were from Gibco BRL (Rockville, MD). Apoptosis detection kit was from BD Pharmingen (San Diego, CA). 2′,7′-dichlorodihydrofluorescein-diacetate (DCHF-DA), dihydroethidium (DHE) and amplex red xanthine oxidase assay kits were purchased from Molecular Probes Inc. (Eugene, OR). SOD (superoxide dismutase), MTT(3-(4,5-dimethyl-2-thiazole)-2,5-diphenyltetrazolium bromide), Xanthine, xanthine oxidase, nitroblue tetrazolium (NBT), Para-hydroxy phenyl acetic acid (pHPA) and horseradish peroxidase were from Sigma Chemicals Co. (St. Louis, MO, USA). MDA (malondialdehyde), SOD, catalase and GST (glutathione-S-transferase) assay kits were from Nanjing Jiancheng Bioengineering Insititute (China). Anti-HIF-1α and anti-SOD were from BD Biosciences Co. (San Jose, CA). HIF-1α ELISA (enzyme-linked immunosorbent assay) kit was from Wuhan USCN Sciences Co., Ltd (China). A secondary antibody (sheep anti-mouse IgG horseradish peroxidase-linked antibody) was from Sino-American biotechnology Co., Ltd (China). Enhanced chemiluminescence substrate was from ECL Plus (Germany). VEGF (vascular endothelial growth factor), IFN-γ (interferon-gamma) and IL-2,4,10 (interleukin-2,4,10) ELISA kits were from R&D Systems (Minneapolis, MN). siRNAs specific to HIF-1α and to SOD were designed and synthesized by GenePharma Company (Shanghai, China). Ac-DEVD-7-amino-4-methyl-coumarine (Ac-DEVD-AMC) was from Biosource International Inc (Camarillo, USA). All other reagents were of analytical grade from commercial sources.

2.2. Animal

Pathogen-free female C57BL/6 and athymic nude mice weighing 18–22 g were purchased from Beijing Weitonglihua Animal Co. All mice were maintained in a pathogen-free animal facility for at least 1 week before each experiment. The animal use committee of Pharmaceutical College of Henan University approved all animal study protocols described in this study, and experiments were conducted in compliance with the guide for the care and use of laboratory animals.

2.3. Cell culture

Human lung carcinoma (A549), mouse lewis lung carcinoma (LLC) and mouse embryonic lung fibroblast (L929) cell lines were purchased from American type culture collection (ATCC; Manassas, VA). Cells were cultured in RPMI1640 medium supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/l glutamine. All cells were incubated at 37 °C with 5% CO_2. Hypoxia treatment was performed by placing cells in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) flushed with a mixture of gas consisting of 1% O_2, 5% CO_2, and 94% N_2.

2.4. Cell proliferation assay

Cells (5×10^3 cells per well) were plated in 96-well plates and allowed to adhere for overnight. Cells were then treated with baicalin at various concentrations or the vehicle (DMSO, final concentration 0.2%) and incubated under normoxia or hypoxia at 37 °C for 72 h. To observe effects of acetylcysteine and catalase on baicalin-induced cytotoxity, cells were cotreated with 10 μmol/l acetylcysteine or 500 units/ml catalase and baicalin for 72 h. Cell proliferation was examined using MTT assay as previously described (Tang et al., 2007), and the IC50, defined as the concentration of drug required to block 50% cell viability, was determined using SPSS software (version 12.0, SPSS Inc, Chicago, IL). VEGF concentrations were determined in supernatants of tumor cells by ELISA according to standard procedures and corrected for cell number.

2.5. Apoptosis assay

Apoptotic and/or necrotic cells were evaluated by Annexin-V binding and PI uptake using an Annexin V-FITC/PI kit as described by the manufacturer. Briefly, tumor cells were plated at a density of 5×10^5 cells/well into 6-well plates for 24 h. The cells were treated with various concentrations of baicalin and incubated under normoxia or hypoxia at 37 °C for 72 h. The cells were washed with cold PBS and resuspended in Annexin V binding buffer. The cells were stained with Annexin V-FITC for 15 min, washed, and then stained with PI. The samples were analyzed by flow cytometer with CellQuest Pro software. SOD, catalase and GST activities were determined in supernatants of tumor cells according to manufacturer's instructions and corrected for cell number.

SOD activity is based on the oxidation of NADH mediated by superoxide radical, one unit of SOD activity is defined as the amount of protein required to obtain half-maximal inhibition of NADPH oxidation. catalase activity is based on the kinetics of 5 mmol/l H_2O_2 degradation at 242 nm, one unit of catalase activity is defined as the quantity that causes reduction of 1 μmol hydrogen peroxide per minute. GST activity is based on the increase in absorbance of the conjugate of GSH and CDNB at 340 nm, one unit of GST activity is defined as the quantity that results in formation of 1 μmol thioether per minute.

2.6. Caspase-3 activity assay

Cells were treated with baicalin as described in apoptosis assay, harvested, and lysed. The cell lysate was centrifuged at 10,000×g at 4 °C for 30 min and the cytosolic supernatant was incubated with Ac-DEVD-AMC according to manufacturer's instructions. The amount of AMC was measured at 460 nm using a fluorescence microtiter plate reader, and the data was expressed as nmol AMC per hour per milligram total cellular protein.

2.7. Transfer of siRNA to cells

Cells (2×10^5) were seeded into 6-well plates on the day before transfection. After 24 h (30–40% confluence), the media was replaced with media without serum or antibiotics. The cells were then transfected with the specific siRNA to HIF-1α or SOD using Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's instructions. After 5 h, the transfection media was replaced with regular complete medium. After 72 h, cells were harvested or treated as needed for further experiments.

2.8. Cellular reactive oxygen species assay

Cells (1×10^4) were seeded in 48-well plates and incubated for 24 h. The cells were treated with various concentrations of baicalin for 72 h, then were washed with phenol red-free HBSS and loaded with 5 μmol/l DCHF-DA or 10 μmol/l DHE for 40 min at 37 °C. Cellular reactive oxygen species and superoxide anion in 10,000 cells were measured using fluorescence intensity of 2′,7′-dichlorofluorescein (DCF) and ethidium respectively by flow cytometry (Mohanty et al., 1997).

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