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Astaxanthin enhances pemetrexed-induced cytotoxicity by downregulation of thymidylate synthase expression in human lung cancer cells

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ABSTRACT

Pemetrexed, a multitargeted antifolate agent, has demonstrated clinical activity in non-small cell lung cancer (NSCLC) cells. Increased expression of thymidylate synthase (TS) is thought to be associated with resistance to pemetrexed. Astaxanthin exhibits a wide range of beneficial effects including anti-cancer and anti-inflammatory properties. In this study, we showed that down-regulating of TS expression in two NSCLC cell lines, human lung adenocarcinoma H1650 and squamous cell carcinoma H1703 cells, with astaxanthin were associated with decreased MKK1/2-ERK1/2 activity. Enforced expression of constitutively active MKK1 (MKK1-CA) vector significantly rescued the decreased TS mRNA and protein levels in astaxanthin-treated NSCLC cells. Combined treatment with a MKK1/2 inhibitor (U0126 or PD98059) further decreased the TS expression in astaxanthin-exposed NSCLC cells. Knockdown of TS using small interfering RNA (siRNA) or inhibiting ERK1/2 activity enhanced the cytotoxicity and cell growth inhibition of astaxanthin. Combination of pemetrexed and astaxanthin resulted in synergistic enhancing cytotoxicity and cell growth inhibition in NSCLC cells, accompanied with reduced activation of phospho-MKK1/2, phopho-ERK1/2, and TS expression. Overexpression of MKK1/2-CA reversed the astaxanthin and pemetrexed-induced synergistic cytotoxicity. Our findings suggested that the down-regulation of MKK1/2-ERK1/2-mediated TS expression by astaxanthin is an important regulator of enhancing the pemetrexed-induced cytotoxicity in NSCLC cells.

1. Introduction

Lung cancer is one of the most lethal malignances in the world, and non-small cell lung cancer (NSCLC) accounts for more than 85% of all lung cancer cases (Ferlay et al., 2010; Pfister et al., 2004; Torre et al., 2015). Pemetrexed is a multi-targeted antifolate drug that inhibits enzymes including thymidylate synthase (TS), dihydrofolate reductase (DHFR) and glycinamide ribonucleotide formyltransferase (GARFT) which are involved in the synthesis of pyrimidine and purine, thereby depleting nucleotide pools and blocking DNA synthesis (Joerger et al., 2010; Tonkinson et al., 1997). Pemetrexed has been approved in first-line, second-line, and maintenance therapy in the treatment of advanced NSCLC (Ciuleanu et al., 2009; Hanna, 2004). In NSCLC, overexpression of TS is associated with poor prognosis following lung resection (Huang et al., 2005; Shimokawa et al., 2011) and low TS mRNA level is associated with better response to neoadjuvant pemetrexed treatment (Bepler et al., 2008). Our previous study has shown that platinum-containing chemotherapeutic compound, cisplatin, increased TS protein expression in a MKK1/2-ERK1/2 dependent manner in NSCLC cell lines (Ko et al., 2011). Moreover, inhibition of heat shock protein 90 (HSP90) can sensitize colorectal cancer cells

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to the effects of chemotherapy through inhibition of ERK1/2 activation and downregulation of TS expression (Nagaraju et al., 2014).

Astaxanthin, a ketocarotenoid (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) red pigment ubiquitously found in microalgae, red yeast, and in marine animals such as shrimp, salmon, crayfish, and lobsters (Goto et al., 2001; Higuera-Ciapara et al., 2006). *In vitro* and *in vivo* studies have reported that astaxanthin possesses several pharmacological properties, including antioxidant, anti-inflammatory, and antitumor effects (Lee et al., 2010; Marin et al., 2011; Nakao et al., 2010; Song et al., 2012). Moreover, astaxanthin induces apoptosis in U937 cells (Human leukemic monocyte lymphoma cell line) by downregulation of AKT activity (Lordan et al., 2008). Furthermore, astaxanthin treatment was shown to promote apoptosis in dimethylhydrazine induced rat colon carcinogenesis through modulating the expressions of ERK, NF-κB, and COX-2 (Nagendraprabhu and Sudhandiran, 2011). Recently, astaxanthin enhances mitomycin C-induced cytotoxic effects in human lung cancer cells lines via AKT inactivation (Ko et al., 2016). However, whether astaxanthin could regulate TS expression to enhance pemetrexed-induced cytotoxic effects in NSCLC has not been examined.

In this study, we wanted to explore the molecular mechanism of astaxanthin in regulating TS expression to enhance the cytotoxic effect of pemetrexed in human lung cancer cells. Using H1650 and H1703 human lung cancer cell lines, we found that decreased TS expression by astaxanthin could enhance the sensitivity of pemetrexed. These results may provide a rationale to combine astaxanthin with pemetrexed for lung cancer treatment.

2. Materials and methods

2.1. Cell lines and chemicals

Human lung carcinoma cells H1650 and H1703 were obtained from the American Type Culture Collection (Manassas, VA) and the cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI-1640 complete medium supplemented with sodium bicarbonate (2.2%, w/v), L-glutamine (0.03%, w/v), penicillin (100 units/ml), streptomycin (100 μg/ml), and fetal calf serum (10%). The cell lines were routinely tested to confirm that they were free of *Mycoplasma*. Astaxanthin (free form) was dissolved in dimethyl sulfoxide (DMSO) immediately before use. Pemetrexed was a gift from Eli Lilly Corporation (Indianapolis, IN, USA). Astaxanthin, Cycloheximide and actinomycin D were purchased from Sigma-Aldrich (St. Louis, MO, USA). N-acetyl-Leu-Leu-norleucinal (ALLN), MG132, U0126, and PD98059 were purchased from Calbiochem-Novabiochem (San Diego, CA, USA). Actinomycin D, ALLN, MG132, U0126, and PD98059 were dissolved in dimethyl sulfoxide (DMSO). Cycloheximide was dissolved in Milli-Q-purified water (Millipore, Billerica, MA, USA).

2.2. Western blot analysis

After cells were treated with pemetrexed (5, 10, 20 μM) and/or astaxanthin (2.5–20 μM) for 24 h, equal amounts (40 μg) of proteins from each set of experiments were subjected to Western blot analysis as previously described (Ko et al., 2009). Antibodies were stripped from polyvinylidene difluoride membranes using a solution containing 2% SDS, 62.5 mM Tris-HCl, pH 6.8, and 0.7% (w/w) β-mercaptoethanol at 50 °C for 15 min before re-probing with another primary antibody. The specific phospho-MKK1/2 (Ser217/Ser221) and phospho-ERK1/2 (Thr202/Tyr204) antibodies were purchased from Cell Signaling (Beverly, MA, USA). Rabbit polyclonal antibodies against TS(TS-106) (sc-33679), ERK2(K-23) (sc-153), HA(F-7) (sc-7392), and Actin(I-19) (sc-1616) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Relative protein blot intensities were determined using a computing densitometer equipped with the ImageQuant analysis program (Amersham Biosciences).

2.3. Plasmid and transfection

Exponentially growing human lung cancer cells (10^6) were plated for 18 h, and then MKK1-CA (a constitutively active form of MKK1, ΔN3/S218E/S222D) and MKK2-CA (a constitutively active form of MKK2, ΔN4/S222E/S226D) were transfected into H1650 or H1703 cells using Lipofectamine (Invitrogen). The sense-strand sequences of siRNA duplexes were as follows: TS: 5′-GCACAUAUUUACCUGAAUC-3′, and scrambled (as a control): 5′-GCG CGC UUU GUA GGA TTC G-3′ (Dharmacon Research, Lafayette, CO). Cells were transfected with siRNA duplexes (200 nM) using Lipofectamine 2000 (Invitrogen) for 24 h.

2.4. Quantitative real-time polymerase chain reaction (PCR)

PCRs were performed using an ABI Prism 7900HT according to the manufacturer's instructions. Amplification of specific PCR products was performed using the SYBR Green PCR Master Mix (Applied Biosystems). For each sample, the data were normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The designed primers in this study were: TS forward primer, 5′- ACTGCAAAGAGTGATTGACACC -3′, TS reverse primer, 5′- CACTGTTCACCACATAGAACTGG -3′; GAPDH forward primer, 5′- CATGAGAAGTATGACAACAGCCT -3′; GAPDH reverse primer, 5′- AGTCCTTCCACGATACCAAAGT -3′. Analysis was performed using the comparative Ct value method. For each sample, the data were normalized to the housekeeping gene *gapdh*.

2.5. MTS assay

In vitro 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was performed. Cells were cultured at 5000 per well in 96-well tissue culture plates. To assess cell viability, drugs were added after plating. At the end of the culture period, 20 μL of MTS solution (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA) was added, the cells were incubated for a further 2 h, and the absorbance was measured at 490 nm using an ELISA plate reader (Biorad Technologies, Hercules, CA).

2.6. Combination index analysis

Pemetrexed and astaxanthin were combined at a ratio of 1:1 or 1:2, and the effect of combined treatment on cell viability was examined by MTS assay. To calculate a combination index (CI), the computer software Calcusyn (Biosoft, Oxford, UK) can be used, taking the entire shape of the cell viability curve into account for calculating whether a combination is synergistic (CI < 0.9), additive (CI = 0.9–1.1), or antagonistic (CI > 1.1) (Peters et al., 2000). The mean of CI values at a fraction affected (FA) of 0.90, 0.75, 0.50 were used to calculate between the three independent experiments.

2.7. Trypan blue dye exclusion assay

Cells were treated with astaxanthin and/or pemetrexed for 24, 48, and 72 h. After treatment, the 500 cells were harvested, and the proportion of dead cells was determined by hemocytometer, counting the number of cells stained with trypan blue. Trypan blue dye can be excluded from living cells, but is able to penetrate dead cells. The dead cells were calculated as follow: trypan blue (+) cells ratio (%) =(stained cell number/total cell number)×100.