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Lycopene inhibits DNA damage and reduces *hMTH*1 mRNA expression in the liver of Mongolian gerbils treated with ferric nitrilotriacetate

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ABSTRACT

Epidemiologic studies suggest that lycopene may reduce the risk of certain cancers, but the underlying mechanisms are unclear. We hereby examined in vivo anticarcinogenic effects of supplemental lycopene on hepatic DNA damage (Comet formation and levels of 8-oxo-2′-deoxyguanosine, 8-oxo-dG) and human MutT homologue (*hMTH*1) mRNA expression in Mongolian gerbils injected i.p. with ferric nitrilotriacetate (Fe/NTA). Gerbils were pre-treated with two dose (10 and 20 mg/kg BW) of lycopene on alternate morning for 10 d. Six hours after the last lycopene supplementation, the gerbils received Fe/NTA (0.16 mmol/10 mL/kg BW) and were killed 3 h later. We found that the livers of Fe/NTA-treated animals exhibited a 5-fold increase in Comet formation (expressed as tail moment), a 2.6-fold increase in 8-oxo-dG levels and a significant increase in *hMTH*1 mRNA expression. Supplemental lycopene completely inhibited Comet formation ($P < 0.001$) and significantly suppressed 8-oxo-dG levels and *hMTH*1 expression in a dose-dependent manner. The expression levels of *hMTH*1 mRNA are highly correlated with hepatic levels of 8-oxo-dG and tail moment, suggesting that *hMTH*1 gene expression represents a molecular marker of oxidative DNA damage. These results demonstrate the potential of lycopene as a promising chemopreventive agent against hepatotumorigenesis.

1. Introduction

Lycopene is an acyclic non-provitamin A carotenoid rich in fruits and vegetables, has been shown to scavenge free radicals and physically quench singlet molecular oxygen, O_2 ($^1\Delta_g$) (Bendich, 1989; Alshatwi et al., 2010). Epidemiological and experimental studies suggest that high lycopene intakes are associated with lowered risk of heart disease, stroke, and several types of cancer (Clinton, 1998; Waliszewski and Blasco, 2010; Wang et al., 2010). Studies have suggested that the anticancer effects of lycopene are related to their effectiveness as antioxidants (Dias et al., 2010; Waliszewski and Blasco, 2010).

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide and one of the leading causes of cancer death in Taiwan. It has been reported that oxidative stress is intricately associated with hepatic carcinogenesis (Marengo et al., 2010). Reactive oxygen species (ROS) are implicated in all stages of carcinogenesis, because they can mutate the DNA, initiate carcinogenesis, and take part in malignant transformation (Halliwell and Gutteridge, 1999). Indeed, oxidative damage to biomolecules such as DNA has been postulated to be involved in many chronic diseases (Ames et al., 1995).

Iron is a well-known inducer of ROS, and the liver is a major site of iron deposits and is, therefore, potentially at a higher risk of cancer development (Agarwal et al., 2007; Matos et al., 2001). Ferric nitrilotriacetate (Fe/NTA) is a potent renal tumor promoter and can cause hepatocellular and renal carcinoma by inducing generation of ROS in primary hepatocytes and mice (Abalea et al., 1998; Agarwal et al., 2007; Ansar et al., 1999; Iqbal and Athar, 1998; Li et al., 1987; Matos et al., 2001). In cultured V79 cells, treatment with Fe/NTA promotes lipid peroxidation, DNA strand breaks, and sister chromatid exchange (Hartwig et al., 1993). Intraperitoneal (i.p.) injection of Fe/NTA to rats increases lipid peroxidation and induces hepatic injury (Lone et al., 2007). Fe/NTA also induces oxidative DNA damage resulting in base-pair mutations or chromosome aberrations, such as 8-oxo-2′-deoxyguanosine (8-oxo-dG), in rat kidney DNA after intraperitoneal administration of Fe/NTA (Toyokuni et al., 1994; Umemura et al., 1990).

Abbreviations: 8-Oxo-dG, 8-hydroxy-2′-deoxyguanosine; BHT, butylated hydroxytoluene; EtBr, ethidium bromide; Fe/NTA, ferric nitrilotriacetate; HCC, hepatocellular carcinoma; *hMTH*, human MutT homologue; LMA, low melting point agarose; LP, lycopene; NMA, normal melting point agarose; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; TBARS, thiobarbituric acid reactive species; THF, tetrahydrofuran; TMOM, tail moment.

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8-Oxo-dG is one of the major products of ROS-induced DNA damage (Kasai and Nishimura, 1984) that has been used as a biomarker of oxidative DNA damage (Kasai, 1997). The misincorporation of 8-oxo-dG is prevented by human MutT homologue (hMTH1), a protein that hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP (Mo et al., 1992). *hMTH*1 has been shown to be overexpressed at the mRNA level in human renal carcinomas (Okamoto et al., 1996), breast tumors (Wani et al., 1998), and lung cancer cell lines (Hibi et al., 1998; Kennedy et al., 1998). Kennedy et al. (2003) found that elevated levels of *hMTH*1 can be detected in the sputum of at-risk individuals, and this marker may be useful for the early detection of lung cancer and to measure the efficacy of chemoprevention trials. Koketsu et al. (2004) indicated that hMTH1 protein expression for DNA damage correlates significantly with aggressive features of colorectal cancer such as the depth of tumor, and lymph node metastasis. However, Jungst et al. (2004) reported that 8-oxo-dG and *hMTH*1 mRNA are lower in non-tumor liver tissues compared to HCC, indicating that hMTH1 acts to prevent 8-oxo-dG-induced mutagenesis.

Although Matos et al. (2001) have found that lycopene inhibits DNA damage and liver necrosis in rats treated with Fe/NTA, it should be noted that they administrated lycopene via ip injection. In addition, it is unclear whether lycopene supplementation affects the expression of *hMTH*1. In this study, we therefore examined the effect of orally-supplemented lycopene on hepatic DNA damage (Comet formation and 8-oxo-dG levels) and on *hMTH*1 mRNA expression in Mongolian gerbils, a rodent species that has been shown to be an appropriate animal model for studying carotenoid metabolism in vivo (Huang et al., 2006).

2. Material and methods

2.1. Chemicals

All chemicals used were of reagent or higher grade. NTA, tetrahydrofuran (THF), nuclease P1, alkaline phosphatase, ribonucleases A and T1, proteinase K, acetonitrile, methanol, and isopropyl alcohol were from Sigma (USA). The lycopene was from Wako (Japan), and the purity was ~98% claimed by the supplier (lot No. 125-04341). The Fe/NTA solution was prepared immediately before use by the method described previously (Matos et al., 2001). Briefly, aqueous solutions of $FeCl_3$ and NTA were mixed at a molar ratio of 1:4 $FeCl_3$/NTA (pH was adjusted to 7.4).

2.2. Animals and diet

Male Mongolian gerbils (8-wk-old, body weights 49–56 g, $n = 40$) were purchased from the animal Center of the National Taiwan University. According to our previously study (Huang et al., 2006), gerbils are better accumulators than nude mice, F344 rats and BALB/c mice, and that the former species may be more useful for studying the in vivo effects of lycopene. The gerbils were housed individually in hanging wire mesh cages with controlled temperature (25 ± 2 °C), humidity (65 ± 5%), and alternating 12 h light:dark cycle. Upon arrival, gerbils were acclimated for 2 weeks, during which they were fed a standard rodent diet (Lab 5001, Purina Mills, St. Louis, MO) and water ad libitum. The standard diet contains 4.5 mg β-carotene/kg but had no detectable amount of lycopene, as indicated by the supplier. The study protocol was approved by the Animal Research Committee of National Chung Hsing University (IACUC Approval No: 96-67).

2.3. Experimental design

Gerbils received orally either corn oil alone (10 mL/kg BW·2 d) as controls or were supplemented with lycopene at 10 (LP10) or 20 (LP20) mg/kg BW in corn oil every other morning (10 am) for 10 d (a total of five dosing). Because the steady state levels of plasma lycopene were achieved after supplementation with 20 mg lycopene/kg BW every other day between 6 and 20 d (Huang et al., 2006), we chose 20 mg and its half level (10 mg) lycopene in the present study. Gerbils were divided into five groups of eight gerbils each: groups I and III were administered orally with corn oil; II and V with LP20; and IV was administered orally with LP10.

On day 10, gerbils in Groups III–V were administered i.p. with Fe/NTA (0.16 mmol/kg BW, given in a volume of 10 mL/kg BW) at 6 h after the last treatment with corn oil or lycopene. After 3 h of Fe/NTA treatment (Matos et al., 2001), gerbils were killed by CO_2 asphyxiation and the livers of each gerbil were immediately removed. Blood samples were collected from both the retro-orbital plexus and heart in a 10 mL vacutainer tube containing K_3EDTA, and were centrifuged (400*g*, 10 min) to separate plasma and store at −80 °C until analysis. A portion of liver was quickly frozen in liquid N_2, and store at −80 °C until use.

2.4. Hepatic lycopene levels

Lycopene was extracted and analyzed using reverse-phase HPLC as described elsewhere (Huang et al., 2006). The tissues were extracted without saponification using 6.0 mL of $CHCl_3$:CH_3OH (2:1, v/v). Retinyl acetate and α-tocopherol acetate were added as internal standards. The tissue mixtures were centrifuged for 10 min at 320*g* at 4 °C, and the chloroform layer was evaporated to dryness under nitrogen, and the residue was redissolved in 15 μL chloroform and 35 μL acetonitrile–methanol mixture (1:1, v/v). A 20 μL aliquot of final extract was injected onto the HPLC system.

Lycopene contents were then analyzed using HPLC, as we described previously (Yeh et al., 2005), which was modified from Su et al. (1999). Absorption maximum of lycopene was at 470 nm. This method determines only lycopene and does not allow the differentiation of isomers. The efficiency of extraction of internal standards ranged from 81% to 87% in tissue samples.

2.5. Preparation of animal tissues for assay of DNA strand breakage (Comet assay)

Comet assay for liver tissues was conducted as we described previously (Chuang and Hu, 2005). Briefly, a portion of the fresh tissue (0.5 g liver) was minced thoroughly on ice, and the minced tissues were added to 10 mL of an enzyme solution containing various amounts of collagenase. The mixture was incubated with shaking (100 rpm) at 37 °C for 20 min followed by low centrifugation (40*g*, 5 min) to remove undigested tissue debris and blood cells. The supernatant was further centrifuged (700*g*, 10 min) to precipitate the cells, which were immediately used for the Comet assay.

2.6. Comet assay

The Comet assay was adapted from the methods of Hu et al. (2002). After isolation, cells were suspended in low-melting-point agarose in PBS at 37 °C and pipetted onto a frosted glass microscope slide pre-coated with a layer of 1% normal-melting-point agarose, the slides were immersed in cold-lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium lauryl sarcosinate, 1% Triton X-100) for 1 h at 4 °C. The slides were then placed in an electrophoresis tank, allowing the DNA to unwind for 15 min in the alkaline solution (300 mM NaOH and 1 mM EDTA). Electrophoresis was then performed at 300 mA for 20 min in the same alkaline solution at room temperature. The slides were then neutralized with 0.4 M Tris–HCl buffer (pH 7.4) and stained with ethidium bromide. Tail moment (TMOM) was calculated by the formula: TMOM = TDNA (% of DNA in tail) × TD (tail distance) using the image Pro Plus software (Media Cybernetics, USA). DNA with a TMOM <2 is considered as normal; 2–5 as minimal; and >10 as middle level of damage because the latter is not readily distinguishable from the former by visual examination (Chuang and Hu, 2005; Hu et al., 2002).

2.7. Measurement of 8-oxo-dG

For assay of nucleic 8-oxo-dG, liver DNA was purified as described previously (Chuang and Hu, 2005). After thawing, liver tissues were homogenized in ice cold 15 mM trisodium citrate/150 mM NaCl, pH 7.0. After centrifuged (10*g*, 5 min) the pellet was washed, centrifuged, and suspended in 50 mM phosphate/150 mM NaCl (pH 7.4). The nuclei thus obtained contained approximately 4.5 mg protein and 1 mg DNA/mL. The nuclear DNA was isolated using phenol/chloroform/isoamyl alcohol (Gupta, 1984). An equal amount of DNA (200 mg) was then digested with nuclease P_1 and alkaline phosphatase, and the 8-oxo-dG levels were analyzed by HPLC with an electrochemical detector (Bioanalytical Systems, model LC-4C) (Shigenaga et al., 1990) as described previously (Hu and Shih, 1997). Oxidative damage was expressed as the molar ratio of 8-oxo-dG to 10^6 molecules of deoxyguanosine (dG), which was calculated from the absorbance at 260 nm.

2.8. RT-PCR (RNA isolation and sequencing)

Total cellular RNA was isolated from hepatic tissues (RNAzol-kit), reverse-transcribed into cDNA (MMLV-Reverse Transcriptase, Gibco/BRL, Bethesda, MD, USA) by using oligo $(dT)_{15}$ as primers and then co-amplified with four primers bases on *hMTH*1 and *β-actin* (internal control) sequences. The primers for amplifying *hMTH*1 cDNA were 5′-CTCAGCGAGTTCTCCTGG-3′, located in the 5′-untranslated region, and 5′-GGAGTGGAAACCAGTAGCTGTC-3′, located in the 3′ untranslated region. In NCBI genome library, only human *hMTH*1 sequence has been cloned. However, the primers that we used are highly reserved in various species including human *hMTH1* sequence among the seven identified genome library. The primers for amplifying *β-actin* cDNA were 5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CTCCTTAATGTCACGCACGATTTC-3′. PCR amplification was performed with a thermal cycler, as follows: denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 90 s, followed by a final incubation at 72 °C for 7 min. The