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Enhancement of DEN-induced liver tumorigenesis in heme oxygenase-1 G143H mutant transgenic mice

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ABSTRACT

Heme oxygenase (HO) is the rate-limiting enzyme in heme metabolism. HO-1 exhibits anti-oxidative and anti-inflammatory function via the actions of its metabolite, respectively. A growing body of evidence demonstrates that HO-1 is implicated in the pathogenesis and progression of several types of cancer. However, whether HO-1 takes part in healthy-premalignant-malignant transformation is still undefined.

In this study, we took advantage of transgenic mice which over-expressed HO-1 dominant negative mutant (HO-1 G143H) and observed its susceptibility to DEN-induced hepatocarcinogenesis. Our results indicate that HO-1 G143H mutant accelerates the progression of tumorigenesis and tumor growth. The mechanism is closely related to enhancement of ROS production which induce more hepatocytes death and secretion of inflammatory cytokines, proliferation of surviving hepatocytes. Our result provides the direct evidence that HO-1 plays an important protective role in liver carcinogenesis. Alternatively, we suggest the possible explanation on effect of HO-1 promoter polymorphism which involved in tumorigenesis.

1. Introduction

Liver cancer is the fifth most frequently diagnosed cancer and the second most frequent cause of cancer death in men worldwide [1]. Most hepatocellular carcinomas are attributed to common risk factors such as hepatitis B or C viral infection and liver cirrhosis or dietary aflatoxin B1 from any cause together with genotoxic and cytotoxic chemical inductions, which usually lead to chronic liver injury and inflammation [2]. A single postnatal injection of the tumor initiator diethylnitrosamine (DEN) can result in hepatocellular carcinoma (HCC) by inducing hepatocyte DNA damage, which can mimic the course of development in human HCC [3].

Heme oxygenase (HO) catalyzes the oxidative degradation of heme to iron, carbon monoxide, and biliverdin. Out of three mammalian HO isoforms, HO-1 is the only inducible one, which is highly expressed in the spleen and liver, and normally found in very low levels in mammalian tissue. HO-1 exhibits anti-oxidative and anti-inflammatory function via the action of its metabolites, respectively [4]. The truncated rat and human HO-1 crystal structure shows that mutation of several amino acids contributes to activity alteration, mutation of Gly139, Gly143 and Gly144 together with His25 exert different effects [5]. Our previous study showed that mouse HO-1 G143H mutants were in competition with wild-type HO-1 to bind heme. HO-1 G143H mutant transgenic mice (HO-1 G143H Tg mice) could mimic HO deficiency *in vivo* and presented with anemia enlarged spleen and tissue iron overload, which was similar to HO-1$^{-/-}$ mice [6].

HO-1 is highly induced in various disease states, including cancer. HO-1 over-expression is commonly seen in several human cancers. A growing body of evidence suggests that HO-1 may play a

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role in tumor induction and can potently improve the growth and spread of tumors [4]. However, other reports also show HO-1 could inhibit proliferation and metastasis in liver cancer [7], breast cancer [8] and prostate cancer [9]. Several genetic studies indicate a high association between HO-1 gene polymorphism and human cancer risk [10–13]. However, there is no any direct evidence that shows the role of HO-1 in tumorigenesis.

Due to low breeding and survival rate, together with short life span, HO-1 knockout mice are not suitable for long term tumor induction. However, HO-1 G143H mutant could mimic lower activity of HO without effect on fertility and survival. Therefore, we investigated the role of HO-1 in DEN-induced hepatocarcinogenesis using HO-1 G143H Tg mice. Our results show that HO-1 G143H Tg mice accelerated DEN- induced hepatocellular carcinoma development.

2. Materials and methods

2.1. Ethics statement

All protocols for animal care and use were approved by the Institutional Animal Care and Use Committee at Harbin Medical University and the Institute of Laboratory Animal Science of China (A5655-01). The animals were handled in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. All the mice were housed in pathogen-free (SPF) animal facilities under a standard 12-h-light/12-h-dark cycle. The animals received free access to water and commercial mouse chow throughout the present study. The mice were sacrificed by cervical dislocation.

2.2. Transgenic mice

Generation of HO-1 G143H Tg mice and littermate wild type (WT) mice were described previously [6]. The background of the transgenic mice was C57BL/6J. The DNA of the mouse tail was extracted according to classical DNA isolation protocol, and genotypes were identified by PCR assay. PCR was performed using the Takara Taq system (Takara Bio, Otsu, Japan) using genotyping primers, sense: 5′-GCCTTCTTC TTTTTCCTACAGCTC-3′; antisense: 5′-GGCATGCTGTCGGGCTGTGGAC-3′.

2.3. Hepatocarcinogen treatment

For hepatocarcinogenesis, 2-weeks-old HO-1G143 H Tg and WT mice of both genotypes were treated with a single intraperitoneal (i.p.) injection of diethylnitrosamine (DEN, Sigma-Aldrich, MO, USA, #73861) at a dose of 25 mg/kg diluted in phosphate-buffered saline (PBS). These mice were used to observe the development of tumors and were sacrificed at 36–45 weeks after initial DEN treatment. For acute liver injury, 8-weeks-old WT mice and HO-1G143 H Tg mice were administrated 100 mg/kg of DEN by i.p. injection. The mice were sacrificed at 24hr or 48hr.

2.4. Serum analysis

Alanine transaminase (ALT) and aspartate transaminase (AST) activity in serum were measured using the ALT and AST Reagent kits with automatic biochemical analyzer.

2.5. Detection of endogenous CO content

The CO content in the blood of HO-1G143 H Tg mice and WT mice were assessed spectrophotometrically by measuring the conversion of deoxyhemoglobin (deoxy-Hb) to carbonmonoxy hemoglobin (HbCO). The blood of transgenic mice were collected and assayed for CO content using endogenous CO detection kit (Nanjing Jiancheng, Nanjing, China) according to the manufacturer's instructions. The amount of HbCO formed was quantified by measuring the absorbance at 540 nm.

2.6. Liver tumor analysis

The whole liver was carefully removed from the euthanized animal, washed, and placed in cold PBS. The number of surface liver tumor nodules was counted for all the liver lobes in a double-blinded manner. Some reasonably sized tumor nodules (≥2 mm in diameter) were carefully removed from the liver lobes using fine forceps and were placed in fresh cold PBS. These separated nodules were then halved using a sterile razor blade and were split into samples for RNA and protein extraction. The body and liver weights, maximum tumor diameter, tumor area were recorded, and tumor incidences, survival rate of mice in each group were calculated.

2.7. Histopathology

The mice livers and nodules were fixed in 10% formalin and embedded in paraffin, partially embedded in OCT used for frozen slice. Tissue sections (4 μm) were prepared and stained with hematoxylin and eosin (H&E) staining according to standard protocols. All sections were visualized using an Olympus light microscope.

2.8. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed as described previously [7]. The oligonucleotide primer sequences used were as follows: IL-6 5′-ACAAATTCGGTACATCCTCG-3′, (antisense) 5′-CTCTGGCTTGTTCCTCACTA- 3′ and β-actin 5′-ACCTCATGAAGA TCCTCACC-3′, (antisense) 5′-TTTCGTGGATGCCACAGGAC-3′. The annealing temperatures for IL-6 and actin were 56 and 60 °C, respectively.

2.9. Western blot analysis

Western blot analyses were performed as described previously [7]. Antibodies from Cell Signaling Technology, Danvers, MA, USA were used to detect PCNA (PC10) (1:2000) (#2586S), phospho-ERK (1:1000) (#4376), ERK (1:1000) (#4696), phospho- Akt (#9271), Akt (#4685), phospho-p38 MAPK (#4511), p38 MAPK (#14451), GAPDH (#2118) were the loading controls.

2.10. TUNEL staining

Quantification of apoptosis of mouse tumor tissues were performed using an In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science, Indianapolis, IN, USA), according to the manufacturer's instructions. Paraffin blocks were cut into slices with 4 μm thickness. The levels of apoptosis were evaluated by counting the TUNEL-positive cells (green signal). The apoptotic index was determined as the number of TUNEL-positive cells/total number of cells in five randomly selected high-power fields (magnification, × 400).

2.10.1. ROS measurement

Fresh frozen samples in OCT were cryo-sectioned to 20 μm, then were measured by the content of ROS in liver tissues using Reactive Oxygen Species in Situ Staining Kit (GMS10124.2, Baoman biotechnology, shanghai) according to the instruction. ROS

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