Study of the Effects of Cyclooxygenase-2 Inhibitor on the Promotion of Hepatic Tumorigenesis in Rats Fed a High Fat Diet

Magda Hamzawy*, Laila Elsaid*, Asmaa Shams*, Laila Rashid†, Soheir Mahfouz‡, Nivin Sharawy*

*Department of Physiology †Department of Biochemistry and ‡Department of Pathology, Faculty of Medicine, Cairo University, Cairo, Egypt

Background/objective: **Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. The highest prevalence of hepatitis is an important risk factor contributing to development of HCCs. However, an increasing number of cases are associated metabolic disease and steatohepatitis. Inflammation associated with many liver disease, seems to be a necessary pre-requisite for successful tumor initiation. Mechanisms that link high fat diet and inflammation initial stage of HCC are not completely understood.**

The present work was designed to investigate the effect of fat, through modulation of the insulin-like growth factors I and II (IGF-I and IGF-II), on the promotion of hepatocellular carcinoma, and the role of cyclooxygenase 2 (COX-2). ***Methods:*** **two main groups of rats were used: control and HCC groups. The HCC group was further subdivide in to two subgroups, HCC fed with standard diet and HCC fed with high fat diet. The effects of celecoxib were also investigated in HCC fed with high fat diet.** ***Results:*** **We found that high fat diet was associated with significant increases in COX2 and interleukin 6 (IL6) with significant promotion of HCC progression. The significant increase in IGF could contribute partially to the observed effects of high fat diet. In addition, celecoxib was found to significantly reduce HCC progression.** ***Conclusions:*** **We conclude that COX2 could play central role in high prevalence of HCC observed with high fat diet. Several triggering factors such as IGF and IL6, together with the direct modulation of fat metabolism could open several novel preventive strategies of celecoxib treatment, and could be useful biomarkers for assessment of its pharmacological effects. (J Clin Exp Hepatol 2015;5:14–21)**

The factors triggering hepatocellular carcinoma (HCC) are thought to be a complex combination involving genetic, infectious, metabolic, environmental and hormonal, which are all involved in complex way. Nutrition represent an important preventable risk factor in development of HCC, with high fat diet enhanced the development of hepatosteatosis, ensuing liver damage and accelerating the appearance of liver tumors.[1,2]

It was presumed that the Insulin-like growth factor (IGF) plays a key role in many neoplastic pathology, such as pediatric tumors, breast and colon cancer, as well as HCC. In addition, tumor cells with non-functional IGF receptors could induce their own proliferation by the synthesis of endogenous IGF molecules. This process of autocrine stimulation contributed in part to the autonomous and faster tumor growth.[3,4]

Growing evidence indicates the inflammatory role of IGF in the initiation and progression of HCC. The important cancer-related inflammatory mediator that is upregulated in most tumors is COX-2, which is documented to be an indicator of poor prognosis in many cancer types.[5,6] In addition, Qiu et al[7] reported that COX-2 may be involved in the early stages of hepatocarcinogenesis. Furthermore, it was reported that COX-2 expression in the tumor tissue was significantly correlated to the presence of inflammatory cells as macrophages and mast cells.[8,9]

In the last few decades, extensive research effort continues to be devoted to the study of the anticancer mechanisms underlying diet modification.[10] It was stated that besides taking steps to avoid hepatitis and cirrhosis, there is no reliable way to prevent liver cancer. However, the

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Address for correspondence: Nivin Sharawy, Kasr El-Aini, Cairo University, Al-Saray Street, 11562 Cairo, Egypt. Tel.: +202 01122433182; fax: +202 23628246
E-mail: nivinsharawi@gmail.com
Abbreviations: AFP: alpha-fetoprotein; CCl4: carbon-tetrachloride; COX-2: cyclooxygenase 2; FAS: fatty acid synthase; FFA: free fatty acid; GH: growth hormone; H&E: Hematoxylin and Eosin stain; HCC: hepatocellular carcinoma; IκB: inhibitory protein; IGFBP-3: insulin-like growth factor-binding protein 3; IGFR: IGF receptor; IGF-I and IGF-II: insulin-like growth factors I and II; IL6: interleukin 6; JNK1: c-Jun N-terminal kinase-1; MAPK: mitogen-activated protein kinase; NFκB: nuclear factor-κB; PI3k: phosphatidylinositide 3-kinases; PAS: periodic acid Schiff stain; real time-PCR: real time-polymerase chain reaction; TAG: triaceyl glycerol
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general cancer prevention guidelines include eating a high-fiber, low-fat diet, exercising, and avoiding alcohol and smoking.[11]

The mechanism linking nutrients to the development of cancer is still not fully understood. We hypothesized that a functional link exists between COX-2, IGF and high fat levels in steatohepatitis associated HCC.

MATERIAL AND METHODS

Animals

After obtaining approval from the institutional Animal Care Committee, we used 48 male albino rats (body weight: 150–200 g, Laboratory Animal House Unit of Faculty of Medicine, Cairo University). The animals were housed in chip-bedded cages and, prior to experiments, acclimated for 1 week in the air conditioned institutional animal care unit. They were housed under 12 h light/dark cycles, with free access to water and standard rat chow.

General Protocol

Male rats were randomly assigned in to one of two groups: control group; represented placebo treated rats and hepatocellular carcinoma (HCC) group. HCC group was further divided in to three subgroups. Subgroup 1; HCC receiving standard rat chow (g/kg): Casein 200.0, Starch 615.0, Cornoil 80.0, Cellulose 50.0, Vitamin–Mineral Premix 50.0, DL-Methionine 3.0, Choline chloride 2.0, Chromium, (mg/kg) 0.066,[12] Subgroup 2; HCC animals received a high fat diet (g/kg): Casein 200.0, Starch 145.0, Sucrose—150.0, Cellulose 50.0, Beef tallow 400.0, Vitamin–Mineral Premix 50.0, DL-Methionine 3.0, Choline chloride 2.0, Chromium (mg/kg) 0.066.[12]

To investigate the role of COX2 in the progression of HCC promoted by high fat diet, subgroup 3 was added; in which HCC animals receiving a high fat diet were orally treated with celecoxib (Sigma aldrich, Cairo, Egypt) (3.6 mg twice/day oral for 6 weeks).

Hepatocarcinogenesis was induced chemically in rats by injecting a single intra-peritoneal dose of diethylnitrosamine (Sigma aldrich, Cairo, Egypt) at a dose of 200 mg/kg body weight, followed by weekly subcutaneous injections of Carbon-tetrachloride (CCl4) (Sigma aldrich, Cairo, Egypt) at a dose of 3 mL/kg body weight for 6 weeks.[13] At the planned time animals were sacrificed by cervical dislocation. The blood samples were collected for further measurement of Alpha-fetoprotein (AFP), Free fatty acid (FFA), triaceyl glycerol (TAG), insulin like growth factor (IGF-I) and insulin like growth factor (IGF-II). In addition, liver samples were dissected for further histopathological assessment using Periodic acid Schiff stain (PAS), Masson's trichrome stain and Hematoxylin and Eosin (H&E) stain. Liver tissue gene expression of Cyclooxygenases (COX-2) and Interleukine-6 (IL-6) were also assessed.

Determination of Serum Alpha-Fetoprotein (AFP)

This was achieved by using rat Alpha-Fetoprotein (AFP) ELISA kit (Kamiya Biomedical company, Gateway drive, Seattle), which applies a technique called a quantitative sandwich immunoassay.

Determination of Serum Levels of Free Fatty Acids (FFA) and Triglycerides (TAG)

The serum FFA and TAG were measured by quantitative colorimetric technique. Biovision's Colorimetric and Fluorometric Assay Kit (BioVision, California, USA) provided a convenient, sensitive enzyme-based detection method, easily quantified by colorimetric (spectrophotometry at λ = 570 nm) in variety samples.

Determination of Insulin-like Growth Factor-I (IGF-I) and Insulin-like Growth Factor-II (IGF-II)

This was achieved by using rat IGF ELISA kit (Boster, Pleasanton, Canada), which was based on standard sandwich enzyme-linked immune-sorbent assay technology.

Real Time-Polymerase Chain Reaction (real time-PCR)

Real time-PCR (Pomega, Madison, WI, USA) was used to measure COX2 and IL-6 gene expression. The key equipment for real time-PCR is a specialized thermocycler with fluorescence detection modules, which is used to monitor and record the fluorescence in real time as amplification occurs. A typical workflow of real time-PCR for gene expression measurement involves RNA isolation, reverse transcription, real time-PCR assay development, real time-PCR experiment and data analysis.

Histo-pathological Examination

Liver samples were fixed in 10% paraformaldehyde and embedded in paraffin. Sections were obtained and stained with hematoxylin and eosin, Masson's trichome stain and Periodic acid Schiff stain (PAS).

Statistical Methods

The results are given as means ± standard deviation (SD). Results were analyzed by using the software Prism 5 (GraphPad Software, La Jolla, CA, USA). Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc test in normally distributed quantitative variables. Correlations were done to test for linear relations between quantitative variables by Pearson correlation. P-values less than or equal to 0.05 were considered as statistically significant.