Chlorella vulgaris triggers apoptosis in hepatocarcinogenesis-induced rats*

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Abstract: Chlorella vulgaris (CV) has been reported to have antioxidant and anticancer properties. We evaluated the effect of CV on apoptotic regulator protein expression in liver cancer-induced rats. Male Wistar rats (200~250 g) were divided into eight groups: control group (normal diet), CDE group (choline deficient diet supplemented with ethionine in drinking water to induce hepatocarcinogenesis), CV groups with three different doses of CV (50, 150, and 300 mg/kg body weight), and CDE groups treated with different doses of CV (50, 150, and 300 mg/kg body weight). Rats were sacrificed at various weeks and liver tissues were embedded in paraffin blocks for immunohistochemistry studies. CV, at increasing doses, decreased the expression of anti-apoptotic protein, Bcl-2, but increased the expression of pro-apoptotic protein, caspase 8, in CDE rats, which was correlated with decreased hepatocytes proliferation and increased apoptosis as determined by bromodeoxy-uridine (BrdU) labeling and terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) assay, respectively. Our study shows that CV has definite chemopreventive effect by inducing apoptosis via decreasing the expression of Bcl-2 and increasing the expression of caspase 8 in hepatocarcinogenesis-induced rats.

Key words: Chlorella vulgaris (CV), Apoptosis, Bcl-2, Caspase 8, Liver cancer
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INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for approximately 85% of the primary malignant tumors of the liver (Kew, 2002). HCC is the fifth most common malignancy in the world and is the third most common cause of cancer-related death worldwide (Okuda, 2000; Kew, 2002). Korea has the highest incidence of liver cancer in the world (Park, 2005). In Malaysian subcontinent, malignant neoplasm (cancer) is the third most common disease after septicemia and cardiovascular disease (National Cancer Registry of Malaysia, 2003). Chronic infection with hepatitis B and hepatitis C, aflatoxin B1, chronic alcohol consumption, and liver cirrhosis have all been implicated in the pathogenesis of liver cancer (Yuspa and Poirier, 1988; Schafer and Sorrell, 1999). Cancer is caused by an imbalance in the rate of proliferation and apoptosis or cell death. Apoptosis can affect the tumor growth in one or more stages in carcinogenesis. Apoptosis is a form of programed cell death characterized by morphological changes in cells executed by cysteine-aspartate proteases (caspases) and regulated by the Bcl-2 family of proteins (Coults and Strasser, 2003; Hanson et al., 2008) involved in the signal transduction pathways.

A good chemopreventive agent is a naturally occurring agent that can induce apoptosis in cancer cell without much side effects (Surh, 1999). Chlorella vulgaris...
**vulgaris** (CV) is a unicellular green microalga that has been widely used for centuries as a food source with complete nutrients, such as carbohydrate, protein, vitamins and minerals, and is marketed commercially as health supplement or incorporated in food such as cereals (Haperin *et al*., 2003). In an animal study, CV has been shown to have anti-atherogenic, anti-cholesterolemic, anti-inflammatory, and antitumor effects (Sano and Tanaka, 1987; Hasegawa *et al*., 2000). It has also been shown to induce apoptosis and oxidative damage in HepG2 cells (Md Saad *et al*., 2006). In the present study, we examined CV as a chemopreventive agent against liver cancer cells via regulation of apoptotic protein, caspase 8 and anti-apoptotic regulator protein, Bcl-2, and correlated these findings with apoptotic rate and proliferation index.

**MATERIALS AND METHODS**

**Animals, chemicals and treatment**

A total of 96 male Wistar rats (200–250 g) were obtained from Animal Care Unit, Universiti Kebangsaan Malaysia (Kuala Lumpur, Malaysia), and were lodged in polycarbonate cages in a room with controlled temperature, humidity, and light-dark-cycle. All experiments were conducted following the guidelines of National Institute of Health for the Care and Use of Laboratory Animals. The study was approved by the Animal Ethics Committee of Faculty of Medicine, Universiti Kebangsaan Malaysia.

Rats were divided into eight groups in terms of diet given, with six rats each. The rats in the control group were given both normal diet and drinking water (normal rat chow from Gold Coin, Malaysia). According to the method of Akhurst *et al*.(2001), the rats in liver cancer-induced group (CDE group) were given choline deficient diet (ICN Biochemicals, USA) supplemented with 0.1% (w/v) ethionine (Sigma Chemical Co., USA) in drinking water. 0.1% (w/v) ethionine was added in drinking water, instead of being supplemented in the pellet, to reduce the risk of mortality upon administration to the rats and to minimize the exposure of the carcinogen. The rats in three groups (CV50, CV150, and CV300) were administered CV alone in three different doses (50, 150 and 300 mg/kg body weight), respectively. They were given 0.1, 0.3, and 0.6 ml of 10% (w/v) CV (100 g of CV diluted in 1 L of hot water) per day via gavage to represent 50, 150, and 300 mg/kg body weight, respectively. The rats in three CDE groups (CDE+CV50, CDE+CV150, and CDE+CV300) were treated with three different doses of CV (50, 150, and 300 mg/kg body weight). The duration of the experiment was 3 months and the rats were sacrificed at 0, 8, and 12 weeks. Animals were anesthetized for liver perfusion procedure prior to excision of the liver. Liver tissue was excised and fixed in formalin and embedded in paraffin for immunohistochemistry work.

**Culture of Chlorella vulgaris (CV)**

Stock of *Chlorella vulgaris* Beijerinck (strain 072) was obtained from the University of Malaya Algae Culture Collection (UMACC, Malaysia) and grown in Bold Basal Media (BBM) (12-h dark:12-h light cycle). The algae were centrifuged 3 times at 3000 r/min for 10 min at 4 °C to separate from the media. The pelleted algae were then diluted in distilled water at three different doses (50, 150, and 300 mg/kg body weight) before use.

**Hematoxylin and eosin (H&E) staining**

Paraffin embedded, formalin-fixed tissues were sectioned at 3-µm thickness and placed on poly-L-lysine covered slides. The sections were deparaffinized and hydrated with sequential washes in xylene and alcohol. The nuclei were stained by immersing in Mayer’s hematoxylin solution (Lab Vision Corp., UK) for 8 min and rinsed under running tap water. The slides were then dipped in 1% (v/v) acid alcohol to remove excess hematoxylin followed by immersing in 2% (w/v) sodium acetate. The slides were rinsed in running water and eosin for 5 min to stain the cytoplasm of the tissue. Finally, the slides were dehydrated through a series of graded alcohols and mounted with dibutylphathalate xylene (DPX).

**Immunohistochemistry staining for Bcl-2**

Bcl-2 was detected by the indirect method using DAKO antigen retrieval solution (DAKO, USA). Mouse anti-human Bcl-2 oncoprotein clone 124 was purchased from DakoCytomation, Denmark. Paraffin-embedded tissues were sectioned into 3-µm thickness. After deparaffinization, the slides were immersed in target retrieval solution (pH 9.0) and heated in water