Modulation of energy metabolic enzyme expression in N-nitrosodiethylamine-mediated hepatocarcinogenesis by Chinese herbs, Huqi San

Zhaoyang Wen, Zhengming Shi, Ping Feng, Xiaowei Xue, Kun Dong and Xuejiang Wang

Abstract. Aim: To elucidate the anticancer mechanism of Huqi San by assessing the expression of G-6-Pase, SDH, ATPase and AFP in N-nitrosodiethylamine-mediated hepatocarcinogenesis in rats.

Methods: A Solt-Farber two-step test model of hepatocarcinogenesis was established by diethylnitrosamine (DEN) and 2-acetylaminofluorene (AAF) in rats to investigate the modifying effects of expression of 6-glucosephosphatase (G-6-Pase), succinodehydrogenase (SDH), adenosine triphosphatase (ATPase) in N-nitrosodiethylamine-mediated hepatocarcinogenesis. Hu Qisan compounded by eight medicinal herbs was prepared in glycoprival granules with which 0.38 g crude herbs/mL solution was prepared for administration. γ-Glutamy-transpeptidase (γ-GT), G-6-Pase, SDH and ATPase were immunohistochemically determined. The activity of alpha-fetoglobulin (AFP) in the livers was meausred using Immunofluorescence.

Results: Huqi San treated rats showed significant decrease in areas of γ-GT positive foci (P < 0.001). On the other hand, the expression of G-6-Pase, SDH and ATPase has obviously altered in Huqi San treated group. The activity of AFP also significantly decreased after the treatment with Huqi San (8 g/kg body weight or 4 g/kg body weight) or total alkali of mistletoe (0.12 g/kg body weight).

Conclusions: Huqi San can obviously increase these activities of G-6-Pase, SDH and ATPase, and at the same time significantly decrease the expression of γ-GT and AFP. Therefore, it can obstruct or inhibit the rat’s liver preneolastic lesion induced by DEN.

Keywords: Chinese herbs, hepatocarcinogenesis, liver preneolastic lesion, metabolic enzyme, diethylnitrosoamine (DEN) hepatocarcinogenesis

Abbreviations: DEN, diethylnitrosamine; AAF, 2-acetylaminofluorene; G-6-Pase, 6-glucosephosphatase; SDH, succinodehydrogenase; ATPase, adenosine triphosphatase; γ-GT, γ-Glutamy-transpeptidase; AFP, alpha-fetoglobulin

1. Introduction

Upregulation of glycolysis, the anaerobic breakdown of glucose to produce ATP, occurs in almost every tumor. Cancer cells seem to increase glycolysis even in the presence of ample oxygen (a process termed
the ‘Warburg effect’), and glycolysis seems to correlate with tumor aggressiveness, indicating that it might be crucial in the evolution of malignancies [20,22,31]. G-6-Pase catalyzes the final reaction steps in both glycolysis and gluconeogenesis. It occurs mainly in glycogenic tissues, such as the liver. There it plays an important role in the synthesis of glucose, a carbohydrate essential for tissue functioning. Glucose is a key substrate for energy production in the human body. Low glucose level in blood stimulates glycogenolysis and gluconeogenesis in the liver and the kidney. G-6-Pase catalyzes a critical step of glucose synthesis, thus playing an important role in the regulation of glucose homeostasis [16]. Any disturbance in this homeostasis may result in a variety of diseases.

Several mitochondrial proteins are tumor suppressors. They include the enzymes of the tricarboxylic acid (TCA) cycle such as SDH and fumarate hydratase. However, to date, the mechanisms the defects of these enzymes in the TCA cycle contribute to tumor formation have not been elucidated. Mitochondria are dynamic organelles that play a central role in cellular metabolism. The primary metabolic function of mitochondria is oxidative phosphorylation, an energy-generating process that couples the oxidation of respiratory substrates to the synthesis of ATP. SDH normally oxidizes succinate to fumarate and two hydrogens donated to reduction site to convert FAD to FADH2 (reduction product). The catalytic activity (succinate oxidation) of this flavoprotein enzyme can be enhanced by flavine adenine dinucleotide (FAD), which also acts as a coenzyme. FAD added to incubation mixtures can accept and donate hydrogen to Coenzyme Q, a component of electron transport chain. Thus, the activity of SDH might be critical in energy metabolism in cancer cells.

Huqi San is a Chinese herbs prescription and was compounded by eight medicinal herbs, they are Ramulus Visci, Radix Astragali seu Hedysari and so on. The primary functions mechanism of Huqi San are in supplementing qi and tonifying the kidney, promoting diuresis, detoxification, and promoting blood circulation to dissolving lumps. On the other hand, Huqi San has achieved remarkable therapeutic effectiveness in the treatment hepatocirrhosis, and also in blockade and reversion of hepatocarcinogenesis. We previously reported that the Huqi San has an obvious effect to block the pathological changes of hepatocarcinogenesis [27–30]. The blocking function was stronger in the initiation stage than promotion. In the present experiments, we observe the regulatory function of Huqi San on the expression of G-6-Pase, SDH and ATPase, and inhibitory effect on the pathologic changes of hepatocarcinogenesis using DEN hepatocarcinogenesis model in rat, for the further study on anticancer mechanism of Huqi San.

2. Materials and methods

2.1. Chemicals

The chemicals used for histochemical reactions were purchased from Sigma Chemical Co., phenazine methosulfate and NBT were purchased from Beijing dingguo biotechnology Co and Tris was purchased from Roche. Antibodies for Immunofluorescence studies were bought from Santa Cruz (Santa Cruz, CA). Total alkali of mistletoe was bought from Beijing Traditional Chinese Medicine Hospital (Beijing, China).

2.2. Animals and treatment

Healthy male Wistar rats (6 weeks old and 135~149 body weight) were purchased from the animal department of Capital Medical University and maintained in a controlled environmental condition of temperature and humidity on alternatively 12 h light/dark cycles. 50 rats were fed standard pellet diet
and water ad libitum. They were random by divided into 5 groups, they are DEN model group, large
dose therapeutic group (8 g Huqi San/kg body weight), small dose therapeutic group (4 g Huqi San/kg
body weight), total alkali of mistletoe group and normal group. The experiments were operated on the
basis of Solt-Farber model. The DEN 200 mg/kg body weight were injected into the abdominal cavity
of experimental rats as an initiation agent. After two weeks, the test entered a selective promotion stage,
there the rat were fed with 0.015% 2-AAF feed. This process went on for 5 weeks. According to the
classical form of operation, we removed the large lobe of liver of rats at the end of third week. Treatment
group began to take Huqi San through the stomach at the first week after removal of large lobe of liver
(PH), this process keep on to till the end of experimental period and the Huqi San was kept supplying
for 4 weeks. Normal group were raised following the old routine. At the end of ninth week, all rats were
sacrificed under anesthesia with pentobarbital after fasting for 24 hours, then the hepatic tissue were
taken at the assigned position that are the right anterior leaf, right back leaf and the caudal leaf, we also
collected blood samples from heart.

2.3. Preparation of Huqi San

Chinese herbal medicine – Huqi San was compounded by eight medical herbs. All the materials ready
to use have the quality matching the Chinese pharmacopia regulation. Huqi San glycoprival granules
were made by Beijing Railway General Hospital. Ramulus Visci (1800 g), Radix Astragali seu Hedysari
(1600 g), Radix Curcumae, (1200 g), Radix Salviae Miltiorrhizae (800 g), Spica Prunellae (800 g),
Semen Persicae (600 g), Semen Cuscatae (1200 g) and Radix Sophorae Flavescentis (800 g) in 70.0 L
of distilled water were gently boiled for 60 min to reduce the volume to 10 L. The herb mixture was
soaked for 1 h at the temperature just below boiling. The decoction was then filtered through delipidated
gauze and then concentrated to ointment under reduced pressure. The ointment was subjected to vacuum
drying to form extractum which was then crushed into to pieces, then granulated and stored in a refrigerator
until use. The Huqi San granules were diluted with water. The concentration of the Huqi San was 0.38 g
dry grains/mL.

2.4. Enzyme histochemistry

2.4.1. Tissue preparations

The tissue specimens were immediately refrigerated by liquid nitrogen for 1 hour, and then were kept
in a refrigerator at −80° for future use. Cryostat sections, were made at −25° using a Leica CM 1850
cryostat, fixed on to 3-Aminopropy-Triethoxy silane-coated slides, and stored at −20° until use.

2.4.2. Histochemical staining for γ-GT positive foci

γ-GT positive foci histochemistry was done by the method of Rutenberg et al. [21]. In brief, the
frozen sections were fixed in acetone (1 h, 4°), homogenized in 50 mM Tris – HCl (pH 7.4, 10 min) and
incubated in a solution of γ-glutamic-acid-4-methoxy-β-naphthylamide as the γ-GT substrate plus Fast
Blue B (1.25 mg and 5 mg in 10 ml incubation medium, respectively) and glycyl – glycine (5 mg in
10 mL incubation medium, pH 7.4) for 30 min at 37°. The sections were then submerged in 100 mM
cuprum sulfuricum (2 mim), washed in normal sodium and mounted in glycopelatin. For the control,
the γ-GT substrate was omitted in the incubation medium. The quantification of γ-GT positive foci was
done by colour video image processor (FW4000-Leica, Germany). The results were evaluated that γ-GT
positive foci are detected as the red petty granules.
2.4.3. Histochemical determination of G-6-Pase

G-6-Pase positive foci histochemistry was done by the method of Rutenberg et al. [21]. In brief, the sodium glucose-6-phosphate was used as a substrate. After incubation for 12 min, at 37°C in 40 ml 0.1 M acetate buffer (pH 6.5) containing 26 mg glucose-6-phosphate, and 1 mL 100 mM Pb(NO₃)₂, the slides were rinsed in distilled water, 0.5% ammonium sulfide for 2 min, and then distilled water. After rinsing in distilled water, the specimens were mounted with glyco-gelatin. The quantification of G-6-Pase positive foci was done using color video image processor (FW4000-Leica, Germany). G-6-Pase activity expressing locus was shown as the brown lead sulfide granules.

2.4.4. Activity of SDH

Activity of SDH positive foci histochemistry was done by the method of Pearse [19]. In brief, cryostat sections were immersed in the incubation medium in 10 ml 0.1 M phosphate buffer (pH 7.8) containing 80 mg sodium succinate dibasic hexahydrate, 1 mg phenazine methosulfate and 10 mg NBT for 10 min at 37°C. All media were freshly prepared just before incubation. After incubation, tissue sections were rinsed in distilled water, and then they were mounted in glyco-gelatin. SDH positive foci are given as the hyacinthine petty granules. These grains were fixed in the mitochondria of hepatocyte peri-plast. The quantification of SDH positive foci was done using colour video image processor (FW4000-Leica, Germany).

2.4.5. ATPase activity

ATPase activity histochemistry was done by the method of Ben Changen and Li Shugen [3]. Cryostat sections were immersed in the incubation medium contained 5 mg Adenosine 5-triphosphate disodium salt, 4 mL 0.1 M Tris-Maleate buffer solution (PH 7.2), 0.6 mL 2% Pb Pb(NO₃)₂, 1 mL 2.5% MgSO₄ and 4.4 mL distilled water for 30 min at 37°C. The slides were rinsed with distilled water, 0.5% ammonium sulfide for 2 min and then with distilled water. After rinsing, the specimens were mounted with glyco-gelatin. The control was prepared in substrate-free media. ATPase activity expressing location was given as brown lead sulfide granules. The quantification of ATPase positive foci was done using color video image processor (FW4000-Leica, Germany).

2.5. Immunohistochemistry

AFP immunohistochemistry was done by the method of M.E. Alpert and Ruth Andrea Seeler [1]. Frozen thin sections were fixed in 4% paraformaldehyde at room temperature for 80 min, blocked by Abidin solution, d-Biotin Solution, 1:10 rabbit serum confining liquid and 5% bovine serum albumin (BSA) for 20 min at room temperature, respectively. And then incubated with AFP goat polyclonal antibody (1:150) at 4°C overnight, then washed with PBS. After that, the sections were treated with labeled rabbit anti-goat antibody (1:150) and SABC- FITC(1:100) for 30 min at room temperature respectively. The sections were washed extensively, the stained tissues were counterstained to see the cellular nucleus using hochest. At last they were all mounted in 90% glycerin. Rabbit serum was used to substitute for the primary antibody as a negative control. The bright green immunofluorescence developed was analyzed under LEICA FW-4000. AFP positive foci were detected as bright green immunofluorescence.

2.6. Statistical analysis

The results are expressed as the mean ± standard deviation (SD). Data analysis was done using one-way ANOVA followed by LSD (Least Significant Difference) test. The differences were considered statistically significant when \( p < 0.01 \).
3. Results

γ-GT is known to be present in adult normal rat bile ducts. But in the case of Carcinogen-induced rat hepatocellular carcinomas, γ-GT activity is highly induced. Induction of γ-GT is an early event in the formation of these tumors and expression of γ-GT is used as a marker to identify preneoplastic cells in these systems. NDEA-induced various sizes of γ-GT positive foci in rat liver in Fig. 1. But No γ-GT positive foci were observed in liver sections of untreated rats (normal group Fig. 1a). In the NDE-hepatocarcinogenesis model rats, various sizes of γ-GT positive foci were noted and the average area of foci was 35.51 (Fig. 1b). Administration of Huqi San (8 g/kg body weight or 4 g/kg body weight) or total alkali of mistletoe group (0.12 g/kg body weight) after DEN exposure significantly reduced the area of DEN-induced big sized foci when compared with model groups (Fig. 1c, 1d, 1e).

The expressions of G-6-Pase, SDH and ATPase were detected histochemically in liver the tissue sections obtained from the rats and the result was summarized in Fig. 2. As shown in Fig. 2-1a, the expression of G-6-Pase, shown as brown staining in the liver was predominantly decreased in the model group (Fig. 2-1b). Both Huqi San (8 g/kg body weight or 4 g/kg body weight) treatment and total alkali of mistletoe (0.12 g/kg body weight), increased the G-6-Pase expression significantly (P < 0.001, compared to model group) (Fig. 2-1c, d, e). As the same way, the enzyme activity of SDH was detected in rat liver, and the results showed that DEN-model group significantly decreased the expression compared to normal control group (P < 0.001), but the SDH expression was significantly magnified (P < 0.01) by Huqi San (8 g/kg body weight) and total alkali of mistletoe (0.12 g/kg body weight) addition (Figs 2, 3). Similar results were also observed in the study of ATPase. ATPase expression was significantly decreased in the model group rats compared to that of normal control rats (Fig. 2) but this decrease was recovered by the addition Huqi San or total alkali of mistletoe (Fig. 2c, d, e).

To localize the expression of AFP, immunofluorescence method was applied to the tissue sections. Expression of AFP was shown as green fluorescence in the cytoplasm and the cell nuclei was stained with blue fluorescence. Every cell was counted randomly in different microscopic fields of each section, and the number of positive cells were evaluated. The AFP positive cell number was 0.65 cells/cm² in the model group, while the positive number of AFP in normal group was only 0.01 cells/cm². After treatment with Huqi San (8 g/kg body weight or 4 g/kg body weight), or total alkali of mistletoe (0.12 g/kg body weight) the positive number of AFP was significantly decreased to 0.19/cm², 0.09/cm², 0.10/cm², respectively, compared to model group (P < 0.001) (Fig. 3).

4. Discussion

Accumulated evidence during recent years indicated that genotoxic carcinogens, including DEN and 2-AAF cause variety of nongenotoxic alterations in cellular physiological processes playing an important role in the tumor formation [2,15] in addition to exerting their genotoxic effects. One of the fundamental events leading to tumor formation is the cell heterotype changes and a lot of juvenile cells are formed [6, 24]. Previous studies on DEN and 2-AAF-induced liver carcinogenesis reported elsewhere indicated the link of the tumor-promoting property of DEN and 2-AAF with inhibition of apoptosis and juvenile cell mult [18,25,26]. Several possible mechanisms have been proposed for the increase of juvenile cell in the livers during DEN and 2-AAF-induced hepatocarcinogenesis, including the altered expression of proteins involved in cellular metabolism [8,14]. In the present study, we observed the abnormal-expression of γ-GT and AFP on DEN and 2-AAF-induced liver carcinogenesis. This was evidenced by the profound increase of γ-GT and AFP expression in the livers of DEN and 2-AAF-treated rats. As, γ-GT is used
Fig. 1. γ-GT expression in rat liver. Liver sections obtained from the animals of normal control (a), model group (DEN + 2-AAF + PH) (b), treated with large dosage therapeutic group of Huqi San (8 g/kg body weight) (c), small dosage therapeutic group of Huqi San (4 g/kg body weight) (d), and total alkali of mistletoe group (e). The black stained areas are the GGT positive foci of various sizes. For details see text.

as a hepato-precancerosis marker [11,32,33], we have proposed that hepatocytes with elevated levels of γ-GT has selective advantage over γ-GT -negative hepatocytes against carcinogenesis when the animal is treated with carcinogens or promoting agents that deplete intracellular glutathione. AFP has been determined a growth factor in both fetal and tumor environment [10,12,13]. Reappearance of AFP in adult serum is often the signals of pathological conditions, particularly the presence of hepatocellular carcinoma (HCC) and germ cell tumors containing yolk sac cell elements [5]. Nowadays, AFP is successfully used as a diagnostic and prognostic tool for HCC. AFP always accompanies with the growth
of liver cells, and it is confirmed that AFP may be related to the proliferation of tumor or fetal cells. The present study reverted, when the hepatocarcinogenesis rats were treated with Huqi San or total alkali of mistletoe, the positive cell numbers of $\gamma$-GT and AFP were significantly decreased compared with model group.

To elucidate the mechanisms responsible for abnormal expression of this AFP and $\gamma$-GT, we investigated the expression of enzymes associated with cellular energy metabolism, which are frequently dysregulated during hepatocarcinogenesis [7]. In hepatocarcinogenesis, the energy metabolism is to be changed, in order to adapt to their rapid proliferation and thus relies on the ATP synthesis by glycolysis even under aerobic conditions rather than by oxidative phosphorylation. Tumor cell mitochondria can differ structurally and functionally from those of normal cells, but clear evidence in favor of this suggestion is lacking. However, there is evidence that some chemical carcinogen primarily attacks mitochondria that is the “molecular clocks” in eukaryotes [9]. Alterations in mitochondrial function, maintenance of ion homeostasis or ATP supply, have been suggested to contribute to cell transformation. The expression of mitochondrial proteins is also altered with less transcription of ATP synthase gene. G-6-Pase~CSDH and ATPase are the enzymes which catalyze the aerobic oxidation of glucose and generate ATP. Analysis of the expression patterns of ATPase, SDH and G-6-Pase in the present experiment showed that these enzyme activities were suppressed in the proliferation in the liver of rats treated with DEN and 2-AAF as was reported elsewhere [17,23]. However, when Huqi San or total alkali of mistletoeis were applied, these enzyme activities were increased significantly compared with the model rats. These results indicated the Chinese herbs Huqi San has improved the mitochondrial function and upgraded the energy metabolism although the detail mechanism is remained unclear. These are associated with the predominant up-regulation of $\gamma$-GT and AFP.
Fig. 3. AFP expression in rat liver. Liver sections obtained from the animals of normal control (a), model group (DEN + 2-AAF + PH) (b), treated with large dosage therapeutic group of Huqi San (8 g/kg body weight) (c), small dosage therapeutic group of Huqi San (4 g/kg body weight) (d), and total alkali of mistletoe group (e). The black stained areas are the AFP activity of various sizes. For details see text.

In summary, Huqi San and total alkali of mistletoe effectively prevented γ-GT positive foci formation in DEN carcinogenesis model as well as regulation of the activities of energy metabolic enzymes and the expression of AFP, and thus provided unequivocal evidence that Huqi San has potential of obstructing hepatocarcinogenesis caused by DEN and 2-AAF. However, further studies are required to understand the precise mechanism of its anticancer effect.

Acknowledgments

This work was supported by the Chinese medicine technology item of Peking City, China. No. JJ 2004–2012 and Beijing Municipal Commission of Education. No. M200610025003.
References


