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Changes in arginase isoenzymes pattern in human hepatocellular carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common tumors worldwide affecting preferentially patients with liver cirrhosis. The studies were performed on tissues obtained during surgery from 50 patients with HCC, 40 with liver cirrhosis and 40 control livers. It was found that arginase activity in HCC was nearly 5- and 15-fold lower than in cirrhotic and normal livers, respectively. Isoenzymes AI (so-called liver-type arginase) and AII (extrahepatic arginase) were identified by Western blotting in all studied tissues, however the amount of AI, as well as the expression of AI-mRNA were lower in HCC, in comparison with normal liver, and those of AII were significantly higher. Since HCC is arginine-dependent, and arginine is essential for cells growth, the decrease of AI may preserve this amino acid within tumor cells. Concurrently, the rise of AII can increase the level of polyamines, compounds crucial for cells proliferation. Thus, both arginase isoenzymes seem to participate in liver cancerogenesis.

Hepatocellular carcinoma (HCC) is one of the most common fatal malignant neoplasm world-wide. The main risk factors for HCC development are liver cirrhosis (about 80% of all cases) and chronic infection with the hepatitis B and C viruses [1]. In early stages liver cancer usually presents with no symptoms. Most hepatocellular carcinomas are first suspected based on the results of CAT or ultrasound scans. There is lack of highly specific marker for the diagnosis of HCC. At present, the most common is alpha-fetoprotein, however its level increases only in about 70% of cases [2,3]. The potentially curative treatment for patients with HCC is surgery. Unfortunately only a small number of patients are suitable candidates for either surgical resection or liver transplantation because of tumor extension and/or poor hepatic function [4].

Liver is the most metabolically active organ. In humans, among the crucial pathways localized in liver, there is the urea cycle. The last enzyme of the cycle is arginase (EC 3.5.3.1) that hydrolyzes L-arginine to urea and L-ornithine. The presence of two distinct genes for arginase has been established. The gene for arginase AI (so-called liver-type arginase) is assigned to chromosome band 6q23 and arginase AII (so-called extrahepatic arginase) at chromosome 14q24.1-24.3 [5,6]. Arginase AI is a cytosolic enzyme that plays a fundamental role in the urea cycle. Arginase AII is a mitochondrial isoform, and its function is not clear. Among all human tissues, liver expresses the highest arginase activity [7]. Thus, changes in arginase activity and/or in the profile of its isoenzymes may reflect liver disorders including carcinogenesis. As yet there are no studies on arginase in hepatocellular carcinoma in humans.

In the current work, we studied arginase activity and the expression of its isoenzymes in tumor tissues obtained from patients with HCC in comparison with cirrhotic and normal liver. We tried to established the possible role of arginase isoenzymes in pathogenesis of this liver-specific tumor.

Materials and methods

Patients and tissues. The study contained tumor tissues obtained from 50 patients with HCC during surgery, samples of cirrhotic liver obtained during liver transplantation from 40 patients with liver cirrhosis, and 40 histologically normal liver tissues (control) removed 6–7 cm from the border of benign or metastatic tumors of patients with haemangioma ($n = 4$), adenoma hepatocellular ($n = 3$), focal nodular hyperplasia ($n = 3$), and colorectal cancer liver metastases (CRCLM, $n = 30$). All patients were treated in the Department of General Transplant and Liver Surgery, at the Medical University of Warsaw. The group with HCC contained 18 females and 32 males with a median age of 57.3 ± 9.1 (range 29–73 years) (T1–T4N0M0, T3N1M0). The group with liver cirrhosis contained 16 females and 24 males, age 44.6 ± 7.2 (range 21–59 years), and the control group 22 females and 18 males, age 56.4 ± 9.8 (range 32–76 years. The patients were diagnosed by increased level of plasma α-fetoprotein (patients with HCC), CEA (patients with CRCLM), ultrasound sonography and computed tomography. The studies were approved by the Bioethics Committee of the Medical University of Warsaw, and informed consent was obtained from all patients.

Methods. Immediately after surgical removal, the tissues were washed in 0.9% NaCl and frozen at −80 °C. The frozen tissue were cut and homogenized in 10 vol. of 50 mmol/l Tris–HCl buffer, pH

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7.5 containing 1 mmol/l $MnCl_2$, 0.2 mol/l KCl and 0.1% (v/v) Triton X-100. After extraction, the homogenates were centrifuged at 12,000*g* for 30 min at 4° C and obtained supernatants were used for arginase activity determination, Western blotting and ion-exchange chromatography.

Arginase activity was measured by determining the increase in the amount of ornithine, according to Chinard [8]. One unit (U) of enzymatic activity was defined as 1 μmol of the product formed per minute at 37 °C, and expressed per 1 g of wet tissue.

Western blotting was performed after electrophoresis in 14% polyacrylamide gel according to Laemmli [9], with rabbit anti-bovine arginase AI and AII polyclonal antibodies (Res. Diagn. Inc., and Santa Cruz Biotech. Inc., USA). Arginase AI from bovine liver and AII from human kidney were used as standards. Blots were visualized using ECL plus Western Blotting Detection System (Amersham).

Ion-exchange chromatography was performed on CM-cellulose to column (10 × 1,5 cm) equilibrated with 20 mM Tris–HCl buffer, 7.5. The not adsorbed arginase AII (anionic isoform) was eluted with the same buffer, and the adsorbed arginase AI (cationic isoform), with a linear KCl gradient (0.5–1.0 mol/l).

Total RNA was isolated from studied tissues by modified method of acid guanidine thiocyanate-phenol-chloroform extraction using TRIzol® reagent (Invitrogen), according to manufacturer's protocol [10]. Expression level of mRNA for arginase AI and AII was defined by reverse transcriptase-polymerase chain reaction (RT-PCR). Specific oligonucleotide primers for arginase isoenzymes were based on nucleotide sequences in NCBI accession nos. for ARGI: NM000045 and for ARGII BC029050, and were as follows: for arginase AI: TGCAACTGCTGTGTTCACTG (forward), TGATGTTG ACGGACTGGACC (reverse), for arginase AII: GTTAGCAGAGCTGTG TCAGA (forward), GGAGTTGTGGTACCTTATCC (reverse). The specific mRNA sequence for β2-microglobulin (housekeeping gene) was amplified as described by Brophy et al.[11], and served as an internal control (forward primer: CCAGCAGAGAATGGAAAGTC, reverse primer: GATGCTGCTTACATGTCTCG). PCR products were separated on 1.5% agarose gel with ethidium bromide. The level of specific mRNA was measured and expressed in semi-quantitative way as the ratio of optical density band of AI and AII to optical density band of β2-microglobulin. The assay was repeated 2 times for each sample and performed in duplicate. System UVI-KS4000, Syngen Biotech. was used for densitometric analysis of Western blotting and RT-PCR results.

Results were expressed as means ± SD. Quantitative comparison between studied groups was performed by Student's *t*-test using Statistica software (StatSoft 6.1) and non-parametric Mann-Whitney *U*-test.

Results

Arginase activity in control liver obtained 6–7 cm from border of 10 benign tumors ranged from 978.4 to 2165.7 U/g wet tissue, with a mean of 1364.3 ± 452.8 U/g, and in tissue removed from the border of CRCLM tumors ranged from 860.4 to 2300.0 U/g (means 1305.8 ± 515.2 U/g). Since the differences between the groups were not statistically significant, the activity of both groups were calculated together and used as the control value, with the mean of 1330.7 ± 480.8 U/g. Significant decrease in arginase activity was observed in cirrhotic liver ($P < 0.001$) with the mean of 520.3 ± 209.2 U/g (range from 147.6 to 888.5 U/g). In HCC arginase activity ranged from 23.2 to 194.6 U/g with the means of 93.4 ± 43.2 U/g (Table 1). The mean activity was nearly 15-fold lower than in control liver and it was independent of the clinical stage of the patients (Fig. 1).

Table 1
Activity of arginase in normal and pathological liver tissues

Tissue	Arginase activity, U/g tissue	
	Range	Means ± SD
Control liver	860.4–2300.0	1330.7 ± 480.8
Cirrhotic liver	147.6–888.5	520.3 ± 209.2
Hepatocellular carcinoma	23.2–194.6	93.4 ± 43.2

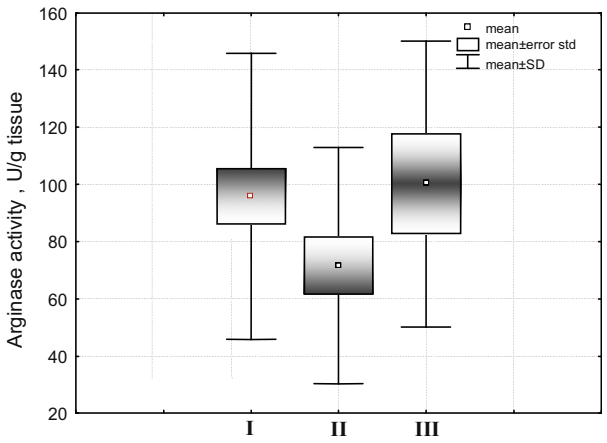

Fig. 1. Arginase activity at different clinical stage of hepatocellular carcinoma. Activity was determined in resected tumors obtained from the patients classified according to IUCC. Group I contained 14 patients (T1N0M0), group II 16 patients (T2N0M0), and group III 20 patients (T3,T4N0M0, $n = 3$; T3N1M0, $n = 17$).

Arginase isoenzymes AI and AII were identified by Western blotting in both control liver and HCC (Fig. 2A and B). When separated by ion-exchange chromatography they contained about 90%

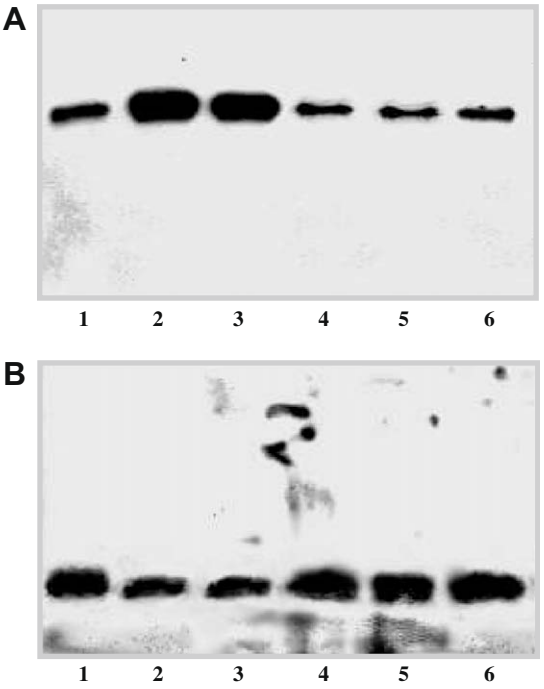

Fig. 2. Western blot analysis of arginase isoenzyme AI (A) and AII (B) in control liver and in hepatocellular carcinoma. Comparable amounts of tissue extract (5 μg of protein) were run in each line, as described in Materials and methods. A: lines 1, 6—standard bovine liver arginase AI; lines 2, 3—arginase AI from control liver removed from the border of benign and metastatic tumors (angioma and CRCLM, respectively); lines 4, 5—arginase AI from hepatocellular carcinomas. B: lines 1 and 6—standard human kidney arginase AII; lines 2, 3—arginase AII from control liver removed from the border of benign and metastatic tumors (angioma and CRCLM); lines 4, 5—arginase AII from hepatocellular carcinomas.