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RESEARCH

Hexokinase II in CD133+ and CD133- Hepatoma BEL-7402 Cells

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Abstract Hexokinase II is a key enzyme in the glycolytic pathway and CD133+ human hepatoma cells possess cancer stem cell-like properties. The expression and enzyme activity of hexokinase II in CD133+ and CD133- hepatoma cells were examined. CD133 on the surface of the hepatoma BEL-7402 cells was analyzed by flow cytometry and the cells were magnetically sorted into CD133+ and CD133- groups. CD133+ cells comprised 1.04% of the total BEL-7402 cell population. Reverse transcription-polymerase chain reaction (PCR) and quantitative real-time PCR were used to assay the expression of hexokinase II mRNA in these two groups. The level of mRNA in CD133- cells was 4.35 times greater than the level in CD133+ cells. 3,6-biphosphoglucose dehydrogenase-coupled colorimetric method and temperature-sensitive trials were applied to determine the enzyme activity of hexokinase II, which was 1.02 U/g protein in CD133+ cells and 2.47 U/g protein in CD133- cells. Hexokinase II was the major active hexokinase isoform in CD133+ cells, comprising 92.7% of the overall cellular hexokinase activity. The results indicate that hexokinase II is vitally meaningful for CD133+ hepatoma BEL-7402 cells. Hexokinase II represents a new therapeutic target for treating CD133+ hepatoma cells.

Keywords Hepatoma · CD133 · Hexokinase II · BEL-7402 · Therapeutic target

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Introduction

CD133 was primitively identified as a hematopoietic stem cell-specific surface marker [1] and found on cells of neural tube, gut, and kidney, murine neuroepithelial cells and several other embryonic epithelia [2]. CD133 has been recognized as a surface marker of organ-specific adult stem cells and cancer stem cells, or tumorigenic cells, including tumors of the brain [3, 4], kidney [5], colon [6, 7], pancreas [8, 9] and prostate [10] and hepatocellular carcinoma (HCC) cells. Suetsugu and other researchers have reported that CD133+ human hepatoma cells possess some cancer stem cell-like properties [11, 12].

Malignant tumor cells often exhibit a high rate of glycolysis. This phenotype was first emphasized by Warburg [13]. Hexokinases play a key role in this metabolic pathway as rate-controlling enzymes. Among these hexokinases, hexokinase II (HK II) is the major isoform and its enzyme activity is remarkably increased in many malignancies [14, 15]. As reported in various malignant tumors and cancer cell lines, when cells become cancerous, the expression and enzyme activity of HK II are up-regulated [16, 17]. HK II is also over-expressed in HCC cells [18–20].

Taken together, the previous findings indicate that glucose metabolism is altered in CD133+ cancer cells. Therefore, we investigated the expression and properties of HK II in the CD133+ cells isolated from human hepatoma cell line BEL-7402. In the end, our research may provide a clue for investigating the glucose metabolism in tumorigenic hepatoma system and establish a basis for an emerging technique to treat HCC, with HK II as the therapeutic target.

Materials and Methods

Cell Line and Culture Conditions

Hepatoma BEL-7402 cells were cultured in RPMI 1640 medium (Gibco, USA) containing 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in 25 cm^2 closed Falcon flasks at 37°C in a humidified incubator at 5% CO_2.

Flow Cytometry

The expression of CD 133 on the surface of BEL-7402 cell was analyzed by flow cytometric analysis (Becton Dickinson FACS Calibur, USA). A total of 1×10^7 harvested cells were incubated at 4°C for 30 min with phycoerythrin (PE)-conjugated anti-CD133/2 antibody (Miltenyi Biotec, Germany) after treatment with FcR blocking reagent (Miltenyi Biotec) to inhibit unspecific or Fc receptor-mediated binding of antibodies to non-target cells. Cell debris and dead cells were eliminated based on scatter signals using 0.5 g/ml propidium iodide (Calbiochem, SanDiego, CA, USA). A total of 20,000 events were acquired for each analysis.

Magnetic Activated Cell Separation (MACS)

About 1×10^8 dissociated cells were centrifuged at 300 g for 6 min at 4°C, rinsed with 300 μl sterile MACS buffer and magnetically labeled with 100 μl CD133 MicroBeads (Miltenyi Biotec). The cell suspension was magnetically sorted using a MACS device. Magnetically labeled CD133+ cells were retained within the column as the positively selected CD133+ cell fraction. Other cells running through the column were acquired as CD133- cells.

Total RNA Isolation and cDNA Synthesis

Total RNA was isolated from the magnetically sorted cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA (2 μg) was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). In addition to 200 units of M-MLV reverse transcriptase, the reaction mixture consisted of 1 μl oligo $(dT)_{15}$ as the primer (Promega), 0.5 units of RNase Inhibitor (Promega), 4 μl RT buffer (250 mM Tris–HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), and 1 μl dNTPs (10 mM, Phamcia), with DEPC-treated water added to a final volume of 20 μl. This mixture was incubated for 5 min at 70°C and then 5 min on ice, followed by 60 min at 42°C and 5 min at 95°C. cDNAs were tested for integrity by amplification of the GAPDH gene. The primers are listed in Table 1.

Table 1 PCR primers used in this study

Primer	Sequence (5′-3′)	Comments
GAPDH F	ACCATGGAGAAGGCTGG GGCTCATT	Forward, Sense
GAPDH R	CATCACGCCACAGT TTCCCGGA	Reverse, Anti-sense
HK-II F	GATTTCACCAAGCGTGGACT	Forward, Sense
HK-II R	CCACACCCACTGTCACTTTG	Reverse, Anti-sense
VDAC-1 F	CGACATGGATTTCGACATTG	Forward, Sense
VDAC-1 R	AGCGCGTGTTACTGTTTCCT	Reverse, Anti-sense

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The HK II gene was analyzed by PCR amplification with specific primers (Table 1). The amplification program included an initial denaturation step at 95°C for 5 min, followed by denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 45 s for 30 cycles. A final extension step was used at 72°C for 5 min. The PCR products were electrophoresed on 1.6% agarose gels and sequenced. Data were analyzed by comparing to the obtained sequences to the Gene Bank. In addition, the VDAC-1 gene, which encodes the HK II binding molecule, was detected using the same procedure.

Real-Time Transcription Polymerase Reaction (PCR)

Quantitative real-time PCR was performed in triplicate using commercially available TaqMan Universal PCR Master Mix, TaqMan probe, and primers (Applied Biosystems, Foster City, CA; GAPDH as control, Hs00266705-g1; HK II, Hs00606086-m1). The procedure was executed on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Forty-two-step cycle amplification was performed with 1 μl cDNA combined with 10 μl TaqMan Universal Master Mix and 1 μl primer pairs and TaqMan probe in a total volume of 20 μl. After activation of AmpliTaq GOLD DNA polymerase at 95°C for 10 min, the amplification procedure was run for 40 cycles under the following conditions: 95°C for 15 s and 60°C for 60 s.

Immunocytochemistry

Several drops of magnetically sorted CD133+ and CD133- BEL-7402 cell suspension was transferred to a 6-well plate with a piece of cover glass at the bottom of the well with the RMPI 1640 as culture medium. After 6 h, when the transferred cells were blown to spread out and adhere to the plate bottom glass, immunostaining was performed to analyze the expression of HK II. After fixation with cold

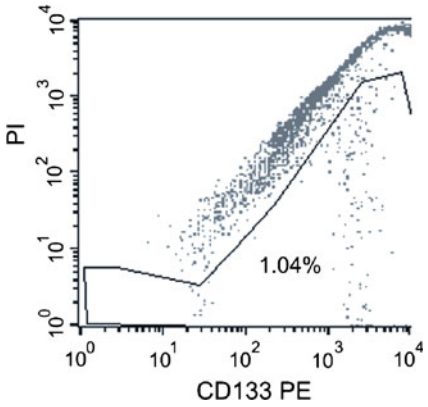

Fig. 1 The expression of surface marker CD133 on the hepatoma cells of BEL-7402 examined by Flow cytometry analysis. The results shown were representative of three independent experiments. PE, phycoerythrin, PI, propidium iodide

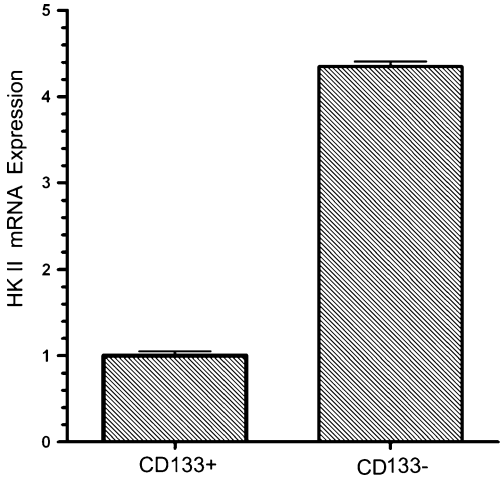

Fig. 3 Quantitative expression of HKII mRNA, with GAPDH as the internal control. The HKII mRNA expression in the CD133- cells was about 4.35 times higher, compared to the CD133+ cells

ethanol, inactivation of the endogenous peroxidase with 3% H_2O_2 and pre-incubation with serum-free blocking solution, 2 μg/ml HK II antibody (Santa Cruz) was added and incubated at 4°C overnight. After washing with PBS, biotin-conjugated mouse anti-goat IgG and horseradish peroxidase-conjugated avidin (Santa Cruz) were applied successively and incubated for 30 min at room temperature for each step and then visualized with 3,3′-diaminobenzidine in H_2O_2 and counterstained with hematoxylin solution.

Hexokinase Enzyme Activity Assay

3,6-biphosphoglucose dehydrogenase-coupled colorimetry method and temperature-sensitive trials [21] were used to test the activities of the overall cellular hexokinase and HK II in CD133+ and CD133- cells. Magnetically sorted BEL-7402 cells were lysed in Triton X-100 lysis buffer (150 mM NaCl, 1% Triton X-100 (Sigma-Aldrich), 50 mM Tris HCl pH 8.0). Some supernatants were tested directly for the overall cellular hexokinase activity and the others were put into a 45°C water bath for 1 h to desensitize HK II because HK II is more thermosensitive. The remaining enzyme activity was then tested. The enzyme activities were determined using the Hexokinase Activity Assay kit (Jiancheng, Nanjing, China) and the procedure conducted by closely following the manufacturer's protocol. The protein concentration was measured using the Brafold Method and the hexokinase activity was calculated using the formula provided by the manufacturer.

Results

Flow Cytometric Analysis of CD133 on the Surface of BEL-7402 Cell

Cultured hepatoma BEL-7402 cells were stained with fluorescence-conjugated primary antibodies against CD133 and then used in flow cytometry analysis. CD133+ cells comprised 1.04% of the total cell population (Fig. 1).

HK II Expression in CD133+ and CD133- Cells

HK II mRNA, as well as that of its contacting molecular, VDAC-1, was expressed in both CD133+ and CD133- BEL-7402 cells (Fig. 2). The length and sequence of the RT-PCR products were consistent with the Genbank sequences.

Quantitative real-time PCR revealed that the HK II mRNA expression was different in these two groups of cells. Setting GAPDH mRNA expression to 100%, HK II mRNA expression in CD133- cells was 1.5%, whereas expression was

Fig. 2 Agarose gel of PCR products of HK II and its binding molecular, VDAC-1, with GAPDH (286 bp) as the internal control. 50 bp marker on DNA ladder was labeled on the *left* side. Bands at 275 bp and 306 bp levels were representative of HK II and VDAC-1, respectively

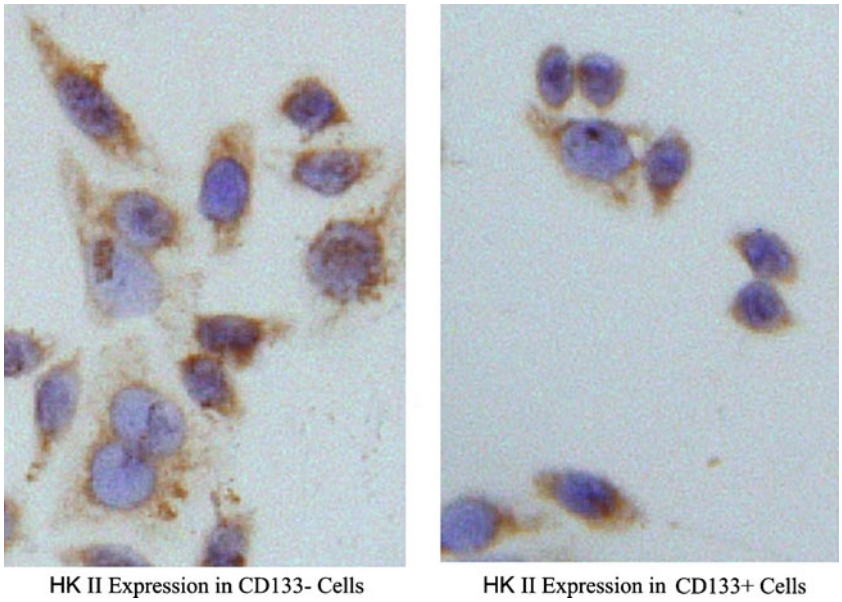

Fig. 4 Immunocytochemistry analysis of HK II in CD 133+ and CD 133- cells. Cells of these two groups were cytoplasmatically stained, with the CD133- cells having stronger signals

0.35% in the CD133+ cells. CD133- cells had approximately 4.35 times the HK II mRNA expression as CD133+ cells (Fig. 3). Immunocytochemistry detected the expression of HK II protein in both CD133+ and CD133- BEL-7402 cells. Cytoplasmic staining was observed in both groups of cells, but greater in CD133- cells (Fig. 4).

Enzyme Activity of HK II in CD133+ and CD133- Cells

Enzyme activity was determined in crude lysis extracts of CD 133+ and CD 133- cells with or without incubation in water bath at 45°C. The water bath protocol was used to discriminate between the activity of HK II and that of the overall cellular hexokinases. Hexokinase enzyme activities present in CD133+ and CD133- cells were 1.10 U/g proteins and 3.80 U/g proteins, respectively, with CD133- cells exhibiting a much higher hexokinase activity. The enzyme activity of HK II in CD 133+ cells was 1.02 U/g proteins, 92.7% of the total overall cellular hexokinase activity. In the CD133- cells the enzyme activity of HK II was 2.47 U/g proteins, 65.0% of the total hexokinase activity (Table 2).

Table 2 Enzyme activities of overall cellular hexokinase and its isoenzyme HK II in CD133+ and CD133- hepatoma BEL-7402 cells

Cell group	Overall cellular HK activity	HK II activity	Percentage
CD133 positive	1.10 U/g proteins	1.02 U/g proteins	92.7%
CD133 negative	3.80 U/g proteins	2.47 U/g proteins	65.0%

Discussion

We found that the CD133 molecule was expressed on the surface of hepatoma BEL-7402 cells, though at a very low level. In addition, the expression of HK II in the CD133- cells was higher than in the CD133+ cells at both the mRNA and protein level. The enzyme activity of HK II was lower in the CD133+ cells, but it comprised the great majority of hexokinase activities for the whole cell. Thus, HK II was almost the only active hexokinase isoform.

CD133 has been implemented successfully in the isolation of human hematopoietic stem cells and other types of stem cells, including endothelial and glial stem cells. Therefore, CD133-selected tumor cells apparently to have some properties of cancer stem cells or tumor initiating cells, which are more metabolically stable and have the characteristics of being self-renewing and multipotent [1, 22, 23]. The enzyme activity of HK II, the only active hexokinase isoform, in the CD133+ cells was relatively lower, indicating that these cells are more metabolically stable. CD133 is the most widely accepted marker, though it is rare, but highly drug resistant [24, 25]. Traditional treatment, such as chemotherapy and radiology, is usually based on the highly active metabolism status of tumor cells, making its effectiveness limited against CD133+ cells. On the other hand, HK II could promote tumor growth through metabolic pathways of glycolysis and pentose phosphate [26, 27] and protect tumor cells from succumbing to apoptosis [28, 29]; thus these CD133+ cells have a great survival advantage. Therefore, our study may provide a rational clue as to why the effect of chemotherapy and radiology for HCC is not obvious, considering the existence of CD133+ HCC cells.

In several recent studies some therapeutic agents against HK II, such as the HK II inhibitor, 3-bropyruvic acid, have been shown to be somehow effective for treating tumors [30–32]. These results indicate that HK II may be promising as a target in treating HCC, especially against CD133+ tumor cells.

Future studies are planned to analyze the functional significance of HK II and other hexokinase isoforms in CD133+ and CD133- hepatoma cells in vitro and in vivo (i.e., using RNA interference and 3-bromopyruvate acid). In addition, samples from clinical settings of human HCC must also be examined to evaluate its potential for clinical application.

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