Effects of small interfering RNA targeting heparanase-1 combined with heparin on invasiveness of mouse hepatocellular carcinoma cell lines

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[Abstract] Background and Objective: Heparanase-1 (HPA-1) can promote angiogenesis and metastasis of malignant tumors and plays an important role in the genesis and development of tumors. This study was to explore the effects of specific small interfering RNA (siRNA) targeting HPA-1 combined with heparin on invasiveness of mouse hepatocellular carcinoma cells. Methods: The expression of HPA-1 in Hca-F, Hca-P, and Hepa-1 cells, which have high, low, and no metastatic potential respectively, was analyzed by reverse transcription-polymerase chain reaction (RT-PCR), Western blot analysis and enzyme-linked immunosorbent assay (ELISA). After transfection with two specific siRNAs targeting HPA-1, siRNA-1 and siRNA-2, and treatment with heparin, invasiveness of Hca-F cells was observed by Matrigel invasion assay. Results: HPA-1 was negative in Hepa-1 cells while positive in both Hca-F and Hca-P cells. The expression levels of both HPA-1 mRNA and protein were obviously higher in Hca-F cells than in Hca-P cells. HPA-1 proteins could be secreted into culture supernatant of Hca-F and Hca-P cells, and the amount of secreted HPA-1 detected by Western blot analysis was larger in Hca-F cells than in Hca-P cells (1.34 ± 0.02 vs. 0.60 ± 0.01, P < 0.001), which was consistent with the results of ELISA. Both siRNA-1 and siRNA-2 downregulated the expression of HPA-1 and the siRNA-2 did more efficiently. The number of invasive Hca-F cells treated with siRNA-2 or heparin alone was larger than that of Hca-F cells treated with combination of them (9 ± 1 vs. 4 ± 1, P = 0.013; 15 ± 2 vs. 4 ± 1, P = 0.008), but smaller than that of untreated Hca-F cells (9 ± 1 vs. 22 ± 2, P = 0.006; 15 ± 2 vs. 22 ± 2, P = 0.026). Conclusion: The combined application of specific siRNA targeting HPA-1 and heparin is more effective in inhibiting the invasiveness of mouse hepatoma cells.

Keywords: Secreted HPA-1, siRNA, heparin, invasiveness

Heparanase-1 (HPA-1), an endoglycosidase, specifically degrades the heparan sulfate side chain of heparan sulfate proteoglycans (HSPG) in the extracellular matrix or at the basement membrane, resulting in the destruction of the extracellular matrix or basement membrane and facilitation of cell movements. HPA-1 also promotes angiogenesis in tumor tissues through the activation of vascular endothelial growth factor (VEGF) that binds to HSPG. Previous studies have revealed that HPA-1 is overexpressed in most tumor tissues including liver cancer, which facilitates angiogenesis and tumor invasiveness, with a positive correlation between HPA-1 expression levels and its effects. Currently, interference methods targeting HPA-1 commonly used are HPA-1 inhibitors and RNA interference (RNAi). Heparin is a complex polysaccharide that inhibits HPA-1 activity. Meanwhile, RNAi technology is widely used to inhibit target gene expression through a specific interaction between small interfering RNA (siRNA) and the target gene. RNAi or heparin alone is able to inhibit HPA-1...
expression and its activity, however, both of them have some disadvantages respectively. RNAi alone cannot completely inhibit HPA-1 expression and high-dose RNAi is also associated with off-target effects. Similarly, anticoagulant effect develops with high-dose heparin. These shortcomings are likely to affect the anti-tumor effects of RNAi and heparin when applied alone. However, the effect of a combination of RNAi and heparin has not been extensively explored.

Hepatocellular carcinoma is one of the most common and malignant cancers. Hepa1-6, Hca-P and Hca-F are three mouse hepatocellular carcinoma cell lines with increasing metastatic ability[6,7]. Studies have shown that the activity of HPA-1 in the blood serum of patients with tumors increased, which is of clinical significance for an early diagnosis of tumor metastasis and prognosis[6]. Therefore, one of the main focuses on research of hepatocellular carcinoma is to study the function of HPA-1 in tumorigenesis and progression, and to look for effective diagnostic markers and treatment for hepatocellular carcinoma by targeting HPA-1. Our study thus aims to determine HPA-1 expression levels in mouse hepatocellular carcinoma cell lines with different metastatic potential, and to investigate the effect of treatment with combination of HPA-1 specific siRNA and heparin on tumor cell invasion.

Materials and Methods

Agents

Hca-F and Hca-P cells were kindly provided by the lab of glycobiology and glycoengineering at Dalian Medical University. Hepa1-6 cell was purchased from Chinese Academy of Medical Sciences; reverse transcription-polymerase chain reaction (RT-PCR) kit was purchased from TaKaRa Bio. Inc., Dalian. Primers were synthesized by TaKaRa Bio. Inc.. Lipofectamine 2000, Protein A-Agarose affinity chromatography, and Trizol were purchased from Invitrogen Corporation. Fetal bovine serum (FBS), RPMI-1640 and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Gibco. siRNAs were synthesized by Shanghai Gene Pharma Co., Ltd. Rabbit anti-mouse HPA-1 antibody, rabbit anti-mouse GAPDH antibody, and horseradish peroxidase-conjugated polyclonal goat anti-rabbit secondary antibody were purchased from Santa Cruz Biotechnology. Heparin was obtained from Tianjin Biochemical Pharmaceutical Co., Ltd. Transwell chambers were purchased from Corning Life Science. Matrigel was purchased from BD Biosciences.

Cell culture

Hca-F and Hca-P cells were cultured in RPMI-1640 medium containing 10% FBS, while Hepa1-6 cells were cultured in DMEM containing 10% FBS. Cells were maintained at a 37°C incubator with 5% CO₂ and cells in logarithmic growth phase were used for experiments.

siRNA design

Two pairs of siRNA (siRNA-1 and siRNA-2), matching sequence domains of 334–353 and 393–411 of HPA-1 mRNA sequence (NCBI access #: NM_152803.4), were designed using the Ambion siRNA software. Scrambled siRNA was also designed and used as a negative control. siRNAs were synthesized by Shanghai Gene Pharma Co., Ltd, and the specific siRNA sequences were as follows: siRNA-1, 5'-TCTCAAGTCAACCATGATATdTdT-3' (forward), 5'-ATATCATGGTTGACTTGAGdTdT-3' (reverse); siRNA-2, 5'-CTCCAGGTGGAATGGCCCTdTdT-3' (forward), 5'-AGGGCCATTCCACCTGGAGdTdT-3' (reverse).

siRNA treatment in Hca-F cells

Hca-F cells were cultured in RPMI-1640 medium containing 10% FBS to reach 60%–80% confluence 24 h before siRNA treatment. siRNA-1 and siRNA-2 were diluted using serum-free RPMI-1640 medium to final concentrations of 10 nmol/L, 30 nmol/L, and 100 nmol/L, respectively. Cells were then transfected with siRNA through Lipofectamine 2000 according to manufacturer’s instruction. Cells were harvested 48 h after transfection for RT-PCR analysis and cell culture supernatants were collected for ELISA assay.

Treatment of Hca-F cells with siRNA-2 and heparin alone or combined

Hca-F cells were seeded to 6-well plates at a density of 3 × 10⁵ cells/well 24 h prior to treatment. The experiment contains 6 groups of cells in triplicates. The siRNA-2 alone group was treated with 10 nmol/L siRNA-2 for 48 h. The combination of siRNA-2 and heparin group was treated with 10 nmol/L siRNA-2 for 48 h, followed by additional incubation with 25 U of heparin for 6 h. The Heparin alone group was incubated with 25 U heparin for 6 h. The combination of scramble siRNA and heparin group was treated with scramble siRNA for 48 h, followed by additional incubation with 25 U of heparin for 6 h. Untreated Hca-F cells were used as a control. Cells in each group were harvested for RT-PCR and the cell culture supernatants were collected for ELISA assay.

Detection of HPA-1 mRNA expression levels using RT-PCR

The sequences of HPA-1 primers were: F1,
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5'-CTTTGGTCTAAATGCCTACTAC-3', and R1, 5'-CTGCCTCATGACGTCTATG-3'. The sequence of GAPDH primers were: F2, 5'-CCAGTGATGAGTCTGCTGAC-3', and R2, 5'-GGATGATGTTCCTGGGACGC-3'. After total mRNA was extracted using Trizol reagent, RT-PCR was performed as described in manufacturer’s instruction. GAPDH served as an internal reference. PCR products were separated in 1% agarose gel. The bands were scanned and relative HPA-1 mRNA expression levels were determined by comparing with the expression of GAPDH.

Detection of secreted HPA-1 in the cell culture mediums using Western blot

The same amount (2 x 10^6) of Hca-F, Hca-P, and Hepa1-6 cells were cultured with 15 mL medium supplemented with 10% FBS for 48 h in 10-cm dishes. Then the cell culture supernatants were collected and purified by Protein A affinity chromatography to remove IgG. The protein contents in the supernatant extract were precipitated with trichloroacetic acid (TCA)-acetone and dissolved in 100 μL loading buffer, then the concentration of protein was measured by Bradford protein assay. A total of 50 μg of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to Western blot analysis. The primary antibody was rabbit anti-mouse polyclonal antibody against HPA-1 (1:200). The second antibody was horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody (1:5000). The immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) kit for Western blot detection. Meanwhile, total proteins were prepared from different hepatocellular carcinoma cell lines and concentration of protein was determined by Bradford protein assay. A total of 50 μg of total protein were separated by SDS-PAGE. The internal control, GAPDH, was analyzed using Western blot analysis. The primary antibody was GAPDH rabbit anti-mouse polyclonal antibody (1:100). The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody (1:8000). The immunoreactive total proteins were visualized by enhanced chemiluminescence (ECL) kit. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control and, after analysis of gel imaging system, relative expression levels of secreted HPA-1 were determined by comparing with the expression of GAPDH.

Detection of secreted HPA-1 in the culture mediums of Hepa1-6, Hca-P, and untreated Hca-F cells by ELISA

The same amount (50 μL) of culture supernatants with IgG removed were obtained from culture mediums of Hca-F, Hca-P, Hepa1-6 cells, and Hca-F cells treated with different concentrations of siRNA-1 or siRNA-2, and the protein contents in the supernatant extract were measured by ELISA assay. Hca-F cells were divided into 6 groups according to different treatment conditions as follows: siRNA-2 (10 nmol/L), siRNA-2 (10 nmol/L) plus heparin (25 U), heparin (25 U), scramble siRNA, scramble siRNA plus heparin (25 U), and untreated control. Five replicates were set for each group. The primary antibody was rabbit anti-mouse polyclonal antibody HPA-1 (1:200) and the second antibody was horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody (1:10000). The absorbance was determined using a microplate reader at 450 nm and the amount of secreted HPA-1 was presented as the mean value of absorbance.

Detection of Hca-F cell migration ability by matrigel invasion assay in vitro

six groups of Hca-F cells described above were cultured in serum-free RPMI-1640 medium for 24 h. Cells were re-suspended in serum-free medium containing 0.1% bovine serum albumin (BSA) at a final concentration of 3 x 10^5 cells/mL. A total of 25 U heparin was added into heparin treated group, siRNA-2 (10 nmol/L) plus heparin treated group and scramble siRNA plus heparin treated group. A total of 50 μg of matrigel was added into each transwell chamber, One hundred μL of cell suspension was added into the upper chambers, and 500 μL culture medium containing 15% FBS was added into the lower chambers. After the cells were cultured in normal condition for 18 h, the polycarbonate membrane was stained by Wright-Giemsa and examined under microscopy. Five high power fields (x 400) of cells were counted and the mean value was calculated as the final result.

Statistical analyses

All data were analyzed using SPSS 12.0 software. The results were presented as mean ± standard deviation (SD) and subjected to t-test analysis. A value of P < 0.05 was considered as statistically different.

Results

Expression levels of HPA-1 mRNA in mouse hepatocellular carcinoma cells with different metastatic potential

RT-PCR results showed that the expression levels of HPA-1 mRNA in Hca-F and Hca-P cells were 1.21 ± 0.02
and 0.25 ± 0.01, respectively, with a significant difference between the two groups (P < 0.001). No HPA-1 mRNA expression was detected in Hepa1-6 cells (Figure 1). These results indicate that HPA-1 mRNA expression levels are positively correlated with the metastatic potentials of mouse hepatocellular carcinoma cells.

Secreted HPA-1 levels in mouse hepatocellular carcinoma cells with different metastatic potential

Western blot analysis showed that the secreted HPA-1 content in culture supernatants of Hca-F cells (1.34 ± 0.02) was significantly higher than that of Hca-P cells (0.60 ± 0.01) (P < 0.001), while no secreted HPA-1 was detected in that of Hepa1-6 cells (Figure 2A). ELISA results also showed that the secreted HPA-1 in culture supernatants of Hca-F cells (0.86 ± 0.03) was higher than that of Hca-P cells (0.21 ± 0.02) (P < 0.001) (Figure 2B). Our results therefore suggest that secreted HPA-1 levels are positively correlated with the metastatic potentials of mouse hepatocarcinoma cell.
Downregulation of the expression levels of HPA-1 mRNA by siRNA-1 and siRNA-2 in Hca-F cells

After Hca-F cells were treated with siRNA-1 at final concentrations of 10 nmol/L, 30 nmol/L, and 100 nmol/L, the expression levels of HPA-1 mRNA were 0.64 ± 0.02, 0.51 ± 0.02, and 0.33 ± 0.02, respectively. After Hca-F cells were treated with siRNA-2 at final concentrations of 10 nmol/L, 30 nmol/L, and 100 nmol/L, the expression levels of HPA-1 mRNA were 0.32 ± 0.01, 0.30 ± 0.01, and 0.20 ± 0.02, respectively. Therefore, the effect of siRNA-2 was significantly greater than that of siRNA-1 (P = 0.002, 0.0008, and 0.008, respectively). siRNA-2 at the concentration of 100 nmol/L worked better than did siRNA-2 at the concentration of 30 nmol/L and 10 nmol/L (P = 0.003 and 0.002), while there was no statistical difference between the 30 nmol/L and 10 nmol/L treated groups (P = 0.070). HPA-1 mRNA expression levels of untreated and scramble siRNA treated Hca-F cells were 0.88 ± 0.01 and 0.87 ± 0.01, which was not significantly different from each other (P = 0.230). These results suggest that HPA-1 specific siRNA-1 and siRNA-2 can effectively inhibit the expression of HPA-1 mRNA in Hca-F cells, and siRNA-2 is more effective. To avoid potential off-target effect of siRNA treatment, we used siRNA-2 at a final concentration of 10 nmol/L for subsequent experiments.

Figure 3  Downregulation of HPA-1 mRNA expression in Hca-F cells after treatment with different concentrations of siRNA-1 and siRNA-2 targeting HPA-1 respectively
A. HPA-1 mRNA was analyzed by RT-PCR assay 48 h after transfection. GAPDH was used as internal control. B, relative signal intensities of HPA-1 mRNA as compared with GAPDH were analyzed by LabWorks (TM ver4.6, UVP, Bioimaging systems)
Lane M, marker; Lane 1, siRNA-1 (10 nmol/L); Lane 2, siRNA-1 (30 nmol/L);
Lane 3, siRNA-1 (100 nmol/L); Lane 4, siRNA-2 (10 nmol/L); Lane 5, siRNA-2 (30 nmol/L);
Lane 6, siRNA-2 (100 nmol/L); Lane 7, RNAi control (scramble siRNA); Lane 8, normal Hca-F cells.

Secreted HPA-1 levels in Hca-F cells treated with siRNA-1 and siRNA-2

ELISA results showed that secreted HPA-1 levels in Hca-F cells were 0.70 ± 0.04, 0.55 ± 0.03, and 0.44 ± 0.03 respectively after treatment with 10 nmol/L, 30 nmol/L, and 100 nmol/L siRNA-1. Secreted HPA-1 levels were 0.41 ± 0.04, 0.39 ± 0.03, and 0.24 ± 0.02 after treatment with 10 nmol/L, 30 nmol/L, and 100 nmol/L siRNA-2 (Figure 4), which were significantly lower than those in siRNA-1 treatment groups (P = 0.024, 0.033, and 0.021, respectively). No significant difference of HPA-1 secretion was observed between 10 nmol/L and 30 nmol/L siRNA-2 treated groups (P = 0.670). In addition, the secreted HPA-1 levels in Hca-F cells after treatment with 10 nmol/L, 30 nmol/L, and 100 nmol/L of both siRNA-1 and siRNA-2 were significantly lower than that in those cells treated with scramble siRNA (0.86 ± 0.03) (P = 0.041, 0.003, and 0.007 for siRNA-1; P = 0.0002, 0.006, and 0.0001 for siRNA-2). No significant difference of HPA-1 secretion was observed between scramble siRNA treated and untreated Hca-F cells (0.88 ± 0.03) (P = 0.570). These results demonstrate that both HPA-1 specific siRNA-1 and siRNA-2 reduce HPA-1 secretion in Hca-F cells and siRNA-2 has a better effect.

Effects of siRNA-2, heparin, or both on the invasiveness of Hca-F cells

In vitro Matrigel invasion assay showed that the number
of invasive cells in siRNA-2 (10 nmol/L) plus heparin (25 U) treated group (4 ± 1) was significantly less than that in siRNA-2 (10 nmol/L) treated group (9 ± 1) and heparin (25 U) treated group (15 ± 2) (P = 0.013 and 0.008, respectively). The number of invasive cells in siRNA-2 (10 nmol/L) treated group was significantly less than that in scramble siRNA treated group (21 ± 1) and untreated group (22 ± 2) (P = 0.002 and 0.006, respectively). The number of invasive cells in heparin (25 U) treated group was significantly less than that in untreated group (P = 0.026). There was no significant difference between scramble siRNA treated cells and untreated normal Hca-F cells (P = 0.225), as well as that between heparin (25 U) treated cells and scramble siRNA plus heparin (25 U) treated cells (15 ± 1) (P = 0.990) (Figure 5). These results indicate that either siRNA-2 or heparin alone can inhibit the Hca-F cell invasion, but the combination of them is more effective.

**Detection of HPA-1 mRNA by RT-PCR in Hca-F cells treated with siRNA-2, heparin, or both**

RT-PCR results showed that the expression level of HPA-1 mRNA in siRNA-2 (10 nmol/L) treated group was 0.30 ± 0.01, which was similar to that in siRNA-2 (10 nmol/L) plus heparin (25 U) treated group (0.31 ± 0.03) (P = 0.480). The HPA-1 mRNA expression level in untreated normal Hca-F cells was 0.92 ± 0.02, which was significantly higher than that in siRNA-2 (10 nmol/L) treated cells (P < 0.001). The expression levels of HPA-1 mRNA in scramble siRNA, heparin (25 U), and scramble siRNA plus heparin (25 U) treated cells were 0.91 ± 0.01, 0.94 ± 0.02, and 0.95 ± 0.03, respectively, which were not significantly different from that in the untreated normal Hca-F cells (P = 0.422, 0.225, and 0.188, respectively) (Figure 6). These results suggest that siRNA-2 inhibits the expression of HPA-1 mRNA, while heparin does not.
ELISA results showed that the amount of secreted HPA-1 in siRNA-2 (10 nmol/L) and siRNA-2 (10 nmol/L) plus heparin (25 U) treated groups were 0.36 ± 0.02 and 0.38 ± 0.04, respectively, with no significant difference (P = 0.225). The amount of secreted HPA-1 in untreated normal Hca-F cells (0.81 ± 0.05) was significantly higher than that in siRNA-2 (10 nmol/L) treated group (P = 0.001). The secreted HPA-1 contents in culture supernatants of heparin (25 U) (0.82 ± 0.02), scramble siRNA (0.79 ± 0.03), and scramble siRNA plus heparin (25 U) (0.82 ± 0.03) treated groups were not significantly different from that of the untreated group (P = 0.729, 0.510, and 0.729, respectively) (Figure 7). These results suggest that heparin does not inhibit the secretion of HPA-1 protein.

**Discussions**

Metastasis is a leading cause of cancer death. Cell adhesion, migration, extracellular matrix degradation and angiogenesis are key factors for tumor metastasis[2]. Studies have demonstrated that HPA-1 plays an important role in tumor angiogenesis and cancer cell invasion, and thus it has become a new therapeutic target to inhibit tumor metastasis.

It has been shown that HPA-1 specific siRNAs can
prevent mouse B16-BL6 melanoma cells from metastasizing to liver and lung, and inhibit angiogenesis in tumor tissues. In addition, B16-BL6 cells which are capable of secreting HPA-1 are prone to metastasize and promote angiogenesis, which can be inhibited by downregulating the secretion of HPA-1 using siRNA\textsuperscript{[1]}. It is generally accepted that secreted HPA-1 enhances cancer cells migration across basement membrane and extracellular matrix, thus facilitating cancer metastasis. However, most of secreted HPA-1 in these studies comes from exogenous expression through DNA recombination and it is difficult to find a cell model with endogenous HPA-1 secretion. In the present study, we found that the expression and secretion of HPA-1 in mouse hepatocellular carcinoma cell lines Hca-P and Hca-F increase as their metastatic potential enhances, and therefore these cell lines provide a good model to study the function of secreted HPA-1 in cancer metastasis.

Two pairs of HPA-1 specific siRNA were used in this study. Since the effect of siRNA-2 is much better than siRNA-1, we chose siRNA-2 in subsequence experiments. We found that there is no significant difference between the 10 nmol/L and 30 nmol/L siRNA-2 treated groups. Even though 100 nmol/L siRNA-2 is more effective, it leads to off-target effect, meanwhile the expression of endogenous GAPDH is also reduced (Data not shown). The reason for this non-specific interference needs further investigation. Therefore, we used siRNA-2 at a final concentration of 10 nmol/L, which inhibits 64% of the expression of HPA-1. Since the remaining 36% of HPA might still exert its function, we explored if other methods can be used at the same time to inhibit HPA-1 activity. Currently, heparin is another important inhibitor of HPA-1 activity. The structure of heparin is similar to the heparan sulfate side chain of heparan sulfate proteoglycans, a substrate for HPA-1. Therefore heparin acts as a competitive inhibitor of HPA-1. It has been confirmed in animal models that heparin inhibits tumor growth, metastasis and angiogenesis through HPA-1\textsuperscript{[2]}. However, it is unclear if the anticoagulant activity of heparin has any influences on its anti-tumor activity, which requires further basic and clinical research. Some studies reported that large doses of heparin may interfere with its clinical application to inhibit tumor metastasis because of the anticoagulant effect. Therefore, it is important to choose an appropriate dose of heparin that can inhibit HPA-1 activity with less anticoagulant activity. To determine the appropriate dose of heparin, we treated Hca-F cells with different doses of heparin and found that the minimum dose of heparin required to decrease the invasion ability of Hca-F cells in matrigel is 25 U (results not shown). Therefore, we used heparin at a concentration of 25 U in this study to inhibit HPA-1 activity.

Our study revealed that the combination of HPA-1 specific siRNA and low doses of heparin can inhibit Hca-F cell invasion more efficiently than does either of them. However, further in vivo experiments are needed to elucidate the effect of the combined treatment on tumor metastasis and angiogenesis. Nevertheless, the combination of HPA-1 specific siRNA and heparin provides a novel idea to treat tumor metastasis and angiogenesis through inhibiting HPA-1 activity.

References