Role of Heparanase-1 in Gastric Carcinoma Invasion

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Abstract

**Purpose:** The heparan sulfate–degrading endoglycosidase may mediate tumor invasion and metastasis. It is known that heparanase-1 (HPA-1) plays an important role in cleaving heparan sulfate. In this study we investigated its potential role in gastric carcinoma malignant behaviour. 

**Methods:** To assess the role of HPA-1, we suppressed its expression using small interfering RNA (siRNA). The human heparanase-1 specific siRNA was transfected to the human gastric carcinoma cell line SGC7901. The effect of gene silencing was assessed by Real-Time PCR and Western Blot. The invasion potential of the cells was evaluated using an in vitro cell invasion model system. 

**Results:** The HPA-1 specific siRNA significantly suppressed expression of heparanase-1 in SGC7901 cells. Invasion after RNAi treatment through membranes in a model system was significantly decreased. 

**Conclusion:** HPA-1 facilitates gastric carcinoma invasion by up-regulating endoglycosidase activity in tumor cells.

**Key Words:** Heparanase-1 - endoglycosidase - siRNA - invasion - metastasis

**Introduction**

Tumor metastasis is the most common cause of death in gastric carcinoma. More than 50% patients die in 2 years after the prognosis of clinical metastasis despite of numerous therapies. It is widely accepted that the tumor metastasis begins with the degradation of extracellular matrix and breaking of basement membrane. Accordingly, only the tumor cells can successfully penetrate the barrier composed by extracellular matrix and basement membrane, and emerge homologous metastasis.

Several specific enzymes, such as heparanase-1 (HPA-1), have been identified, and they can degrade extracellular matrix, facilitating the processes of invasion and metastasis of gastric carcinoma. Heparan sulfate proteoglycans (HSPG), composed of a protein core covalently linked to heparan sulfate side-chains, are the principle component of the extracellular matrix and the external surface of cell membranes (Turnbull et al., 2001). HPA-1, HSPG specific degrading endoglycosidase, cleaves heparan sulfate side chains of HSPG. This process facilitates tumor cells’ migration by degrading the extracellular matrix and basement, leading to mobilization and release of a number of bioactive molecules, including growth factors, chemokines, cytokines, and enzymes that are essential for angiogenesis, cell adhesion, and locomotion (Bernfield et al., 1999; Esko and Selleck, 2001). A predominant correlation was observed in heparanase-1 expression with tumor invasion and lymphatic metastasis in gastric carcinoma (Endo et al., 2001; Takaoka et al., 2003).

RNA interference is a powerful tool of mRNA knockdown to suppress the target gene expression. In this study, we studied the effect of HPA-1 on invasion of gastric cancer cell line SGC7901 by siRNA. 

**Materials and Methods**

**Materials**

Nitrocellulose membrane filters were purchased from Millipore (Bedford, MA); Matrigel was from Becton Dickinson Company (New Jersey, USA); Fibronectin was from Sigma(USA); Transfection reagent Lipofectamine 2000 was from Invitrogen (Carlsbad, USA); Monoclonal anti-human heparanase-1 antibody was from Insight (Rehovot, Israel); SYBR Premix Ex Taq (Perfect Real Time) was from TaKaRa (Otsu, Japan); FCS and RPMI 1640 were from Gibco (Carlsbad, USA); SuperScript® First-Strand Synthesis kits for RT-PCR were from Invitrogen (Carlsbad, USA). 

**Cell line and cell culture**

The SGC7901 cell line, established from gastric carcinoma metastatic lymph node, was from Research Center of Medical Genomics of China Medical University. These cells were cultured in RPMI 1640 medium containing 10% heat-inactivated FCS at 37°C with 99% humidity and 5% CO2, and subcultured every 3 day.

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Double-stranded siRNA and negative control

The sequence of HPA-1 targeted double-stranded siRNA was designed by the online tool of Ambion Company according to the Reynolds principle. The nucleotide sequences of double-stranded siRNA were 5’-GAACAGCAGCACCACUCAAAGAUU-3’ and 5’-UCUUUGAUAGGUGUUCUU-3’ respectively. A control siRNA duplex, 5’-AACCTGGATGGTCTGTCCTGG-3’ and 5’-CAGTCAGGACACTCAAGAGCTTGTCCTG-3’, was used with the same contents of A, T, G, C. Both siRNA were chemically synthesized by GenePharma (Shanghai, China), and stored at -20°C until use.

siRNA Transfection

SGC7901 cells with the confluence of (30-50) % were transfected with either the Heparanase-1 siRNA or the control siRNA using LipofectamineTM 2000 reagent (Invitrogen) according to the manufacturer’s protocol. After 48 hours and 72 hours of siRNA transfection, cells were subjected to extraction of total RNA and protein for quantification.

RNA Extraction and Semi-quantitative Reverse Transcription-PCR

For cell RNA extraction, total RNA was extracted using Trizol (Invitrogen) 48 hours after siRNA transfection according to the manufacturer’s protocol. Briefly, total RNA (100ng/µl), primer, deoxyligionucleotide triphosphate (dNTP) (1 mmol/L), Random hexamers (5ng/µl), and DEPC-treated water were mixed together to a total volume of 10µl, and then were incubated at 65°C for 5 minutes, followed by incubation on ice for 2 minutes. The reaction mixture and 1µlRT buffer (50 mmol/L), MgCl2 (2.5 mmol/L), and DTT (50 mmol/L) were mixed and incubated at 25°C for 2 minutes. After addition of 1 µl (50 units) of SuperScript II RT to each sample, they were incubated at 25°C for 10 min. Samples were transferred to 42°C and incubated for 50 minutes. Reactions were terminated by increasing temperature to 70°C for 15 minutes. PCR amplification with specific primers was performed in a final reaction volume of 20 ml containing 1µlPCR buffer, 200 mmol/L dNTP, 0.5 mmol/L primers, 0.5U of Taq polymerase (Takara Biochemicals, Japan), and 2µl of RT product. Programmable temperature cycling (UNO II, Biometra, Germany) was performed using the following profile: 95°C for 1 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s. After the last cycle, an elongation step of 10 min at 72°C was added. The following primers were used:(a) heparanase-1, forward 5’-CACAAAACCTGCAAAATCACAAGG-3’, and reverse 5’-CCATTGAGTTGGAAGAGTTTG-3’ (PCR product, 180bp); (b) GAPDH, forward 5’-GTGATGGTAGCTGCTTTGTG-3’ and reverse 5’-TACAGCAACAGGGTGTTGGA-3’ (PCR product, 227 bp). The level of mRNA was quantified with Eagle eyeTM II analysis system.

Quantitative Real-Time PCR Analysis

The Real-Time PCR detection with specific primers was performed on the Rotor Gene 2000 Real-Time PCR Cycler (Corbett Research) based on the SYBR Green I fluorescence methodology system. A standard curve was established with TA cloning vector containing the same fragment amplified by the specific primers for HPA-1 or GAPDH. To normalize the quantity and integrity of total RNA used for cDNA synthesis, we amplified the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase(GAPDH). The final reaction volume of 25_1 contained 1µSYBR Premix Ex Taq (TaKaRa), 0.5 µmol/L primers, 1µl of RT product and dH2O. The temperature cycling was performed using the following profile: 95°C for 10s, followed by 34 cycles of 95°C for 5s, 55°C for 20 s. The following primers were used:(a) heparanase-1, forward 5’-CACAAAACCTGCAAAATCACAAGG-3’, and reverse 5’-CCATTGAGTTGGAAGAGTTTG-3’ (PCR product, 180bp); (b) glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5’-TACAGCAACAGGGTGTTGGA-3’ and reverse 5’-TCCACACCCCTTTGTGCTGTA-3’ (PCR product, 130bp).

Western Blot Analysis

Cells cultured in six-well plate were washed by PBS and harvested with RIPA (Tris 50mmol/L, NP-40 1%, NaCl 150mmol/L, EDTA 1mmol, SDS 0.1%, SDC 0.25%). The protein concentrations of the cell homogenates were quantified using the Bradford assay. The samples were subjected to SDS-PAGE and transferred electrophoretically onto a nitrocellulose membrane. The membranes were blocked with 5% nonfat dry milk and 0.1% Tween in TBS (Tris-Cl 50mmol/L, NaCl 150mmol/L), then incubated with mouse monoclonal anti-human heparanase-1 antibody. The anti-mouse IgG antibody coupled with horseradish peroxidase was used as a secondary antibody. The resulting bands were detected using an enhanced chemiluminescence Western blotting detection system according to the manufacturer’s protocol. We performed the immunoblot analysis for GAPDH as a reference in the similar way using the mouse anti-GAPDH polyclonal antibody and the anti-mouse IgG antibody coupled with horseradish peroxidase as primary and secondary antibodies, respectively.

Invasion Assay

Invasion of SGC7901 cells was measured by numbers of cells through Matrigel-coated millicell inserts (Millipore, USA.). The millicell inserts of 8-µm pores were coated with matrigel on the upside (40µg per well) and with fibronectin on the downside by passive adsorption (5µg per well). After transfected with HPA-1-targeted siRNA or control siRNA, the cells were cultured in RPMI 1640 medium containing 10% FCS for 72 hours. Then cells (2µl105 per 100µL per well) were transferred into the upper chamber and allowed to invade for 12 hours. Noninvasive cells were removed from the upside of membrane with a cotton swab. Migrating cells attached to the downside of the membrane were fixed, stained with hematoxylin and eosine, and then examined under microscope. A minimum of 10 random fields (×400) per
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Insert were counted. All the experiments were done independently and in triplicate.

Statistical Analysis

Statistical analysis was carried out with One-Way ANOVA test using the SSPS11.0. P < 0.05 was considered to be statistical significance.

Results

HPA-1 knock down by RNAi

We assessed the effect of siRNA on HPA-1 knockdown at both mRNA and protein expression level. Cells with the confluence of (30-50) % were treated with various doses of HPA-1 siRNA, ranging from 5 to 40nM. We found that HPA-1 siRNA significantly decreased the level of HPA-1 mRNA (Figures 1 and 2) and protein (Figure 3) in a dose-dependent manner.

Role of HPA-1 in tumor invasion

HPA-1 plays a critical role in the progress of tumor invasion. To explore the effect of HPA-1, we conducted the in vitro invasion assay through knockdown of HPA-1 by siRNA. Data showed decreased invasion of SGC7901 cells treated with HPA-1 siRNA. As shown in Figure 4, the cell intensity of HPA-1 knockdown SGC7901 cells was significantly lower than that of the blank control (BC-nontransfection) and negative control (NC-control siRNA).

Table 1. Results of in vitro Invasion Assay for Three Independent Experiments

<table>
<thead>
<tr>
<th>N</th>
<th>Cell Count (cells/field)</th>
<th>BC</th>
<th>NC</th>
<th>HPA-1 Knockdown</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>63 ± 8</td>
<td>28±3</td>
<td>19 ± 9</td>
<td>&lt;0.001 0.028</td>
</tr>
<tr>
<td>2</td>
<td>54 ± 23</td>
<td>43±20</td>
<td>20 ± 15</td>
<td>0.696 0.019</td>
</tr>
<tr>
<td>3</td>
<td>36 ± 16</td>
<td>26±17</td>
<td>7 ± 13</td>
<td>0.332 0.010</td>
</tr>
</tbody>
</table>

P1, BC vs NC; P2, NC vs HPA-1 knockdown

Discussion

Heparan sulfate and heparin sulfate proteoglycans, which are important structural components of the extracellular matrix and the external surface of cell membranes, play a major role in cell-cell and cell-extracellular matrix interactions. They are also functioned as a tissue barrier against the invasion of tumor cells. The cleavage of heparan sulfate chains allows cell growth, migration, and angiogenesis by releasing heparan sulfate-bound cytokines and growth factors, such as basic fibroblast growth factor, from cell surfaces and extracellular matrixes (Vlodavsky et al., 1991). Human HPA-1, an endoglycosidase, is specifically involved in cleaving heparan sulfate, which is related to the invasion and metastasis of cancer cells (Hulett et al.,1999; Kussie et al.,1999; Toyoshima and Nakajima,1999; Vlodavsky et al., 1999). The activity of HPA-1 closely correlates with tumor invasion, metastasis (Marchetti et al., 2000; Nakajima, 1992; Walch and Marchetti,1999; Zhang et al., 2007) and inflammatory reactions (Bartlett et al.,1996; Mollinedo et al., 1997). HPA-1 is up-regulated in metastatic cancers. Heparan sulfate–degrading endoglycosidase activity associated with metastatic tumor cells was first identified in mouse B16-F10 melanoma cells cultured on an extracellular matrix of vascular endothelial cells (Nicolson et al.,1983).

Based on previous studies, we speculated that HPA-1 is involved in the invasion and metastasis of gastric
cancers (Wang et al., 2005; Wang et al., 2005). In this study, a good correlation was observed between the heparan sulfate-degrading endoglycosidase activity and gastric carcinoma’s invasion and lymphatic metastasis. In fact, overexpression of HPA-1 was intensively associated with greater tumor invasiveness including Borrmann gross type and depth of wall infiltration (Wang et al., 2005). We found the expression of heparanase in prime gastric mucosa and metastatic lymph node, indicating its role in gastric metastasis. Of the laser capture microdissected primary gastric carcinoma cells, 47% (14/30) were HPA-1 positive (Wang et al., 2005). Of metastatic cell groups dissected from lymph nodes, 95% showed clear HPA-1 expression. Furthermore, the extent of lymphatic spread was directly correlated to HPA-1 expression at the primary site. Similar results were obtained in other studies. It was reported that 52% (25/48) patients with gastric cancer had a detectable heparanase-1 expression, and the positive rate was 100% (48/48) and 59% (28/48) for the cases with clinically evident of peritoneal metastasis and serosal invasion (Wang et al., 2005). Taken together, HPA-1 can be regarded as a marker of invasion and metastasis in gastric cancer.

In this study, we found that RNAi dramatically reduced the expression of HPA-1 in SGC7901 cells, which significantly inhibited the cell invasion. RNA interfering, targeting critical molecules involved in cancer cell invasion, could be applied in treatment of tumor metastasis. For instance, liver metastasis of colorectal cancer was strongly suppressed by pretreatment of the cells with siRNA targeted macrophage migration inhibitory factor (MIF) (Sun et al., 2005). This study may provide the basis for HPA-1 as a new target in treatment of tumor cell invasion and metastasis in gastric cancer.

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References


