Down-regulation of heparanase leads to the inhibition of invasion and proliferation of A549 cells in vitro and in vivo

Zhitao Chen, Liangming Zhu, Xiaohua Li, Hui Tian, Yusong Fang, Haibo Liu, Shuhai Li, Lin Li, Weiming Yue, and Wenjun Li

1Department of Thoracic Surgery, Jinan Central Hospital Affiliated to Shandong University, Shandong University, Jinan 250013, China
2Department of Thoracic Surgery, Qilu Hospital, Shandong University, Jinan 250012, China
*Correspondence address. Tel: +86-531-82169463; Fax: +86-531-86927544; E-mail: tianhuiy@sohu.com

Heparanase is a mammalian endoglycosidase that degrades heparan sulfate at the cell surface and in the extracellular matrix. The expression of heparanase was detected in a wide variety of human malignant tumors and closely associated with tumor invasion, metastasis, and angiogenesis. However, the specific roles of heparanase and its mechanisms of regulating the malignant potential of non-small cell lung cancer (NSCLC) cells still remain unclear. In the present study, the expression of heparanase was down-regulated in NSCLC cell line by antisense oligodeoxynucleotide. Results showed that down-regulation of heparanase led to significant inhibition of invasive and proliferative potentials of A549 cells in vitro and in vivo. Further research demonstrated that down-regulation of heparanase significantly inhibited the angiogenic potential of A549 cells, which might be the mechanism responsible for the inhibition of A549 cell proliferation in BALB/c nude mice in vivo. These findings demonstrate that heparanase plays essential roles in regulating the invasion, proliferation, and angiogenesis of A549 cells.

Keywords: non-small cell lung cancer; heparanase; antisense oligodeoxynucleotide; invasion; proliferation

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Introduction

Tumor invasion and metastasis depend on the ability of cancer cells to invade tissue barriers in a process involving degradation of the basement membrane (BM) and extracellular matrix (ECM) [1,2]. Heparan sulfate (HS) chains interact through specific attachment sites with the main protein components of BM and ECM, such as collagen IV, laminin, and fibronectin. In addition, HS binds various cytokines and growth factors, such as basic fibroblast growth factor, hepatocyte growth factor, and insulin-like growth factor, and serves as a reservoir for these factors [3–5]. Heparanase is an endoglycosidase capable of specifically degrading HS. Consequently, it facilitates migration of tumor cells, and elicits an angiogenic response by releasing HS-bound angiogenic growth factors from ECM and BM [6–9]. So heparanase has been considered as an attractive target for cancer therapy [10]. Many polyanionic compounds capable of inhibiting heparanase enzymatic activity have anti-tumor and anti-metastatic activities [11–13]. However, the use and mode of action remain questionable due to lack of specificity [14]. In recent years, antisense technology has emerged as an exciting and promising strategy for cancer therapy. The principle of antisense technology is the sequence-specific binding of an antisense oligonucleotide to the target mRNA, resulting in the prevention of gene transcription. The specificity of hybridization makes antisense treatment as an attractive strategy to selectively regulate the expression of genes involved in the pathogenesis of malignancies [15].

Non-small cell lung cancer (NSCLC) is one of the most common malignancies and has been the leading cause of cancer-related death worldwide. Despite the major advances in surgery, radiotherapy, and chemotherapy over the recent decades, the prognosis of patients remains relatively poor due to advanced disease, local relapse, distant metastasis, and resistance to adjuvant therapy [16]. Therefore, exploration of effective and better-tolerated therapeutic strategies is clinically needed.

Here, we investigate the functions of heparanase in regulating the invasive and proliferative potentials of human NSCLC A549 cell line by antisense oligodeoxynucleotide (ASODN) in vitro and in vivo.

Materials and Methods

Cell line and culture conditions

Human NSCLC cell line A549 was purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China).
China) and cultured in RPMI 1640 medium (Hyclone, Logan, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO, New York, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. A549 cells were incubated at 37°C with 5% CO₂ in a humidified cell culture incubator.

Design and synthesis of ASODN
Sequences of ASODN (5'-GGCTTCGAGCGCAGCAGC A1-3') were designed to be complementary to the initial coding region of heparanase mRNA. It is verified that ASODN can only match the corresponding sites of heparanase gene by Blast in GenBank. The oligonucleotide sequence was synthesized, purified, thiophosphorylation-modified, and fluorescein-labeled by Shanghai Sangon Biotech Co., Ltd (Shanghai, China).

ASODN transfection
The ASODN transfection was performed using Lipofectamine™ 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions. Briefly, A549 cells were seeded in a six-well plate and incubated until it reached about 80% confluence. Then cells were washed twice with phosphate-buffered solution (PBS) and RPMI 1640 medium was added to each well. The ASODN and Lipofectamine™ 2000 were mixed gently and incubated together in RPMI 1640 medium to form ASODN-Lipofectamine complex at room temperature for 30 min. Cells were incubated in the medium containing ASODN-Lipofectamine complex at 37°C with 5% CO₂ in a humidified cell culture incubator for 6 h, and then the medium was replaced with RPMI 1640 medium containing 10% FBS. The cells were incubated for another 48 h, and harvested for western blot analysis of heparanase protein expression. Different concentrations of heparanase ASODN (100, 200, and 400 nM) were used to detect the effect on heparanase expression, respectively. A549 cells cultured in RPMI 1640 medium supplemented with only 10% FBS were used as the negative controls. The transfection efficiency was evaluated by fluorescence microscopy.

Western blot analysis
Cells were lysed in lysis buffer (Beyotime, Nantong, China) at 4°C for 30 min, and the lysates were centrifuged (12,000 g) at 4°C for 20 min. The supernatants were collected and the protein concentrations were detected using the enhanced bicinchoninic acid protein assay kit (Beyotime). Total protein (25 μg) was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and then electro-transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Boston, USA). After being blocked with 5% fat-free milk in Tris-buffered saline Tween-20 (pH 7.6), the PVDF membranes were incubated with primary antibodies against heparanase (1:500, sc-25825; Santa Cruz Biotechnology, Santa Cruz, USA) and β-actin (1:1000, AA128; Beyotime) overnight at 4°C. Then the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Signals were detected using the Chemilucent ECL detection system (Millipore). Optical band density was quantified by the Image J software version 1.43 (NIH, Bethesda, USA).

Heparanase activity
Cell lysates prepared from 1 × 10⁶ cells by three cycles of freezing and thawing in pH6.6 heparanase reaction buffer (20 mM phosphate-citrate buffer containing 1 mM dithiothreitol, 1 mM CaCl₂, and 50 mM NaCl) were incubated for 3 h at 37°C, with 3⁵S-labeled ECM. The incubation medium was centrifuged at 20,000 g at 4°C for 15 min, and the supernatant containing 3⁵S-labeled HS degradation fragments was eluted by gel filtration on a Sepharose CL-6B column (0.9 cm × 30.0 cm). Fractions (0.2 ml) were eluted with PBS, and the radioactivity was measured with a β-scintillation counter. Degradation fragments of HS side chains were eluted from Sepharose 6B at 0.5 < Kav < 0.8 (fractions 20–30). Each experiment was performed three times, and the variation in elution positions (Kav values) did not exceed ±15% of the mean. Lysates from untreated A549 cells as controls were incubated with 3⁵S-labeled ECM, and the incubation medium was analyzed as described above.

Transwell invasion assay
Transwell chambers (6.5 mm diameter inserts, 8.0 μm pore size, polycarbonate membrane; Costar, Lowell, USA) of 24-well plate were used in this assay. The membrane at the bottom of each chamber was evenly coated with 50 μl of 1 mg/ml Matrigel (BD, Franklin Lakes, USA). The cells were collected and resuspended in serum-free RPMI 1640 medium at a concentration of 5 × 10⁵ cells/ml by cell counting for three times. Then the cell suspensions were added into the top chambers (200 μl/well) and the bottom chambers were filled with RPMI 1640 medium containing 10% FBS (600 μl/well). After being cultured at 37°C for 24 h, the cells without penetrating the polycarbonate membrane were wiped off with cotton bud. The membrane was removed and fixed with methanol and stained with eosin solution. Five fields were randomly selected under a ZX81 microscope (Olympus, Tokyo, Japan) and the number of cells was counted.

Animal experiments
Six-week-old female BALB/c nude mice were obtained from the Institute of Zoology, Chinese Academy of Sciences (Beijing, China). All procedures were carried out in accordance with the advice and permission of the Institutional Ethical Committee of Shandong University (Jinan, China).
A549 cells were firstly transfected with heparanase ASODN before being injected into the mice. After being cultured in RPMI 1640 medium supplemented with 10% FBS for 24 h, cells were collected and prepared as single-cell-type suspensions (5 × 10^6 cells in 0.1 ml PBS). In control groups, heparanase ASODN was replaced by PBS. Cells were injected subcutaneously at the same site of the back of nude mice (eight mice per group). Mice were examined weekly and tumor growth was evaluated by measuring the length (L) and width (W) of tumor mass with a caliper. Tumor volume (V) was determined by the equation: \( V = L \times W^2 \times 0.5 \). Palpable tumors at the injection sites with a size of more than 3 mm in diameters were monitored as a tumor. Finally, mice were killed by cervical dislocation, and tumor masses were dissected, weighed, and fixed in 10% neutral buffered formalin solution for histological examination.

**Immunohistochemical staining for CD34**

All tumor masses fixed in 10% neutral formalin were embedded in paraffin at 55°C, and serial tissue sections were cut at 4 μm intervals. Briefly, the endogenous peroxidase activity was blocked with 3% H2O2 in methanol for 20 min. The sections were permeabilized with ethylene diamine tetraacetic acid buffer solution (pH 9.0) for 15 min with microwave. Then, the blocking serum was applied for 30 min to prevent the non-specific adherence of serum proteins followed by incubation with primary antibody (1 : 100, sc-19621; Santa Cruz Biotechnology) at 37°C for 2 h. After being washed with PBS, sections were then incubated with biotin-labeled secondary goat anti-mouse HRP IgG antibody (1 : 100; AbD Serotec, Kidlington, UK) at 37°C for 30 min and washed with PBS. After the addition of peroxidase-conjugated streptavidin for 20 min, sections were washed with PBS. Finally, sections were developed with 3,3′-diaminobenzidine solution and counterstained with hematoxylin. For the negative control, PBS was used instead of primary antibody.

**Analysis of microvessel density**

Microvessel density (MVD) was assessed by counting the CD34-positively immunostained endothelial cells according to the international consensus on the methodology and criteria of evaluation of angiogenesis quantification in solid tumors [17]. Briefly, three most vascularized areas were initially selected (so-called ‘hot spots’) under ×40 field. Then, microvessels were counted in each of these areas under ×200 field. Any yellow-brown immunostained endothelial cell or endothelial cell cluster that was clearly separated from adjacent microvessels was considered as a single countable microvessel. The mean value of three ×200 field counts was recorded as the MVD of the section.

All counts were performed by two investigators in a blinded manner. The immunostained slides were re-evaluated jointly by both investigators on a second occasion under a multihead microscope in the cases of contradictory scores and reached a consensus score.

**Statistical analysis**

All statistical analyses were performed using SPSS 15.0 software package. Statistical significance was assessed by comparing mean values (mean ± SD). A value of \( P \) less than 0.05 is considered statistically significant.

**Results**

**ASODN down-regulated heparanase expression**

The transfection efficiency of ASODN at three different concentrations was all more than 70%. Western blot analysis was carried out to examine the down-regulatory effect of ASODN on heparanase expression in A549 cells. Results showed that ASODN at different concentrations all resulted in significant down-regulation of heparanase expression compared with the negative control in a dose-dependent manner (Fig. 1).

**ASODN inhibited heparanase activity of A549 cells in vitro**

To measure heparanase activity, lysates from A549 cells transfected with ASODN or PBS (control group) were
incubated with $^{35}$S-labeled ECM, and the $^{35}$S-labeled degradation fragments released into the incubation medium were analyzed by gel filtration. Compared with the control group, A549 cells transfected with three different concentrations of ASODN had a marked decrease in heparanase-mediated degradation of HS (55%, 62% and 73%, respectively; Fig. 2), demonstrating that the heparanase enzymatic activity was efficiently inhibited by the ASODN in a dose-dependent manner.

**Down-regulation of heparanase inhibited invasion of A549 cells in vitro**

To evaluate the association between heparanase expression and invasive potential of A549 cells, the matrigel invasion assay was performed in a modified Boyden chamber system. The number of A549 cells treated with different concentrations of heparanase ASODN (100 nM, 23.5 ± 0.82; 200 nM, 12.5 ± 0.75; and 400 nM, 5.8 ± 0.55) penetrated to the lower side of the polycarbonate membrane was significantly smaller compared with the negative control (48.5 ± 1.22; Fig. 3). In addition, the inhibitory effect was dose dependent significantly.

**Heparanase ASODN inhibited the proliferation of A549 cells in vivo**

To investigate the effect of heparanase ASODN on the proliferation of A549 cells in vivo, the kinetic growth of A549 cells was assessed in BALB/c nude mice. All 16 mice survived with single subcutaneous tumor formed. As shown in Fig. 4(A), injection of A549 cells transfected with heparanase ASODN (400 nM) induced a marked reduction in the incidence and volume of tumors in the mice compared with those in the control group. The average weight of tumors in the control group was 1744 ± 110 mg, whereas the ASODN group (400 nM) developed significantly smaller tumors with an average weight of 813 ± 66 mg [Fig. 4(B)].

**Heparanase ASODN inhibited tumor angiogenesis in vivo**

To further explore the possible mechanism by which heparanase ASODN inhibits the proliferation of A549 cells in vivo, intratumoral MVD was quantified by counting CD34-positive endothelial cells. Statistical analyses showed that MVD in the tumors of mice injected with A549 cells transfected with heparanase ASODN (13.5 ± 1.8) was significantly less than that in the control group (24.7 ± 2.6; Fig. 5).

**Discussion**

In the present study, to selectively block heparanase expression, an antisense strategy was used. ASODNs are such a class of new approach—they are short, synthetic stretches of DNA, which hybridize with specific mRNA strands that...
correspond to target genes. By binding to the mRNA, the ASODN prevents the sequence of the target gene from being converted into protein, thereby blocking the action of the gene. The specificity of this mechanism has resulted in a new class of drugs with a wide range of potential clinical applications [15,18]. Our results showed that both heparanase expression and its enzymatic activity were significantly inhibited. Several groups have reported that inhibition of heparanase by means of antisense strategy or gene silencing has resulted in decreased invasiveness of cancer cells both in vitro and in vivo [19–21]. Consistent with these previous reports, our results demonstrated that the invasion potential of A549 cells transfected with ASODN was significantly inhibited, indicating that heparanase plays important roles in regulating tumor progression. Thus, approaches that selectively block the heparanase expression implicated in cellular invasion may be more efficacious in preventing cancer cell dissemination. However, the non-enzymatic functions of heparanase such as cell migration and adhesion, which are not sensitive to the currently available heparanase inhibitors, were not investigated in the present study. Edovitsky et al. [19] found that heparanase-mediated cell adhesion was markedly inhibited in lymphoma cells by using siRNA-mediated gene silencing strategy but not by laminaran sulfate, which efficiently inhibited heparanase enzymatic activity. Further investigation is required to evaluate the functions of heparanase in regulating the adhesion and migration potentials of A549 cells by using specific gene silencing strategy in vitro, which might thus provide novel insights into the functions of heparanase (i.e. cell migration and adhesion) unobtainable by other methods of inhibiting heparanase enzymatic activity.

In vivo experimental results showed that BALB/c mice subcutaneously injected with A549 cells transfected with ASODN resulted in a much lower tumor incidence, smaller tumor volume and lighter tumor weight than those in the control group. It is well known that angiogenesis is an early event in carcinogenesis and plays essential roles in the progression. To explore the possible mechanism by which heparanase ASODN inhibits the proliferation of A549 cells in vivo, intratumoral MVD was quantified by counting...
CD34-positive endothelial cells. Our results demonstrated that heparanase ASODN was able to suppress angiogenesis in the nude mice model of A549 cells. The anti-angiogenic effect of heparanase ASODN is primarily the result of an indirect effect of heparanase down-regulation on the bioavailability of angiogenesis-promoting factors that are sequestered by HS and can be released by the tumor-derived heparanase [6,7,9,22]. Thus, heparanase-related angiogenesis might play important roles in promoting NSCLC progression and development of aggressive phenotypes, and heparanase might be a potential novel therapeutic target for anti-angiogenesis in NSCLC patients. However, the exact mechanisms by which heparanase promotes angiogenic potential of A549 cells are not yet known and still need to be further elucidated at the molecular level. We intend to reduce heparanase expression by RNAi in NSCLC cells, to investigate the relationships between heparanase expression and major angiogenic factors both in vitro and in vivo. Such studies will provide new and important clues to explore the mechanisms of heparanase involved in the regulation of angiogenesis in NSCLC.

In conclusion, our results showed that down-regulation of heparanase expression by ASODN led to the significant inhibition of invasive potential of A549 cells in vitro, as well as the proliferative and angiogenic potentials of A549 cells in vivo. These findings provide new insights and potentially clinical utility for heparanase as a novel potential therapeutic target for NSCLC patients by inhibiting invasion and angiogenesis of cancer cells.

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References