RESEARCH COMMUNICATION

Heparanase Antisense Suppression of A-549 Lung Carcinoma Invasion

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Abstract

Objective: Heparanase (HPSE), as the only enzyme which can degrade the extracellular matrix and heparin sulfate in basement membrane, plays an important role in invasion and metastasis of tumor cells. In this study, we evaluated the inhibitory effect of HPSE antisense oligoxydeonucleotide (ASODN) on lung carcinoma cell line A-549 invasion. Methods: Liposome-mediated ASODN was transfected into A-549 cells and expression of HPSE protein and mRNA were detected by flow cytometry and RT-PCR techniques. Matrigel invasion assays were employed to assess effects on invasiveness. Results: Lower expression of HPSE protein and mRNA and lower invasive ability to recombinate basal membrane were apparent after ASOND treatment (P<0.01). The inhibition rates of cell invasiveness were 55.6%, 82.3% and 91.2% treated by ASODN at final concentrations of 100, 200 and 400nmol/L, respectively. Conclusions: HPSE ASODN can downregulate the expression of HPSE protein and mRNA in the A-549 cell line and can obviously inhibit its invasive ability in a dose-dependent manner in vitro.

Keywords: Lung carcinoma - heparanase - antisense oligoxydeonucleotide - in vitro

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Introduction

In recent years, multidisciplinary treatment of lung cancer has reached a new level, but long-term treatment is still unsatisfactory. Research suggests that treatment failure is primarily due to tumor recurrence and metastasis. Tumor invasion and metastasis, as the most essential biological characteristics, are related directly to poor prognosis and mortality of patients. HPSE can specifically degrade the extracellular matrix and heparin sulfate proteoglycans of basement membrane, and promote invasion and metastasis directly and indirectly (Vlodavsky et al., 1999). In this study, HPSE ASODN was transfected into A-549 cell lines, its invasive ability and expression of HPSE were examined, maybe the HPSE will be a new target gene in treatment of lung cancer.

Materials and Methods

Human lung cancer cell line A-549 cells were purchased from Shanghai Cell Institute of Chinese Academy of Sciences, RPMI-1640 culture medium was from Gibco BRL Company. A-549 cells were maintained in RPMI-1640 with 10% Fetal bovine serum. Cells were in the logarithmic growth phase. According to HPSE gene sequence (GenBank accession number: AF 155510), ASODN sequence was designed to complement to start codon region of HPSE mRNA (AUG and its downstream 17 nucleotide sequence), and conformed that ASODN sequence only matches the corresponding sites of the gene HPSE. The sequence above was synthesized by Shanhai Bio-Engineering Company, pured and phosphorothioate-modified. End bases were labeled by fluorescein isothiocyanate. Sequence of ASODN is 5'-GGCTTCGAGCGCAGCAGCA -3'. Three groups were divided into according to different transfection complexes: control group (blank control group), LIP group (lipofectamine transfection), and ASODN group(ASODN transfection). According to the pre-test results, transfection rate is high in these three different final concentrations 100,200,400nmol/L, there is no significant difference in three concentrations above.

The 6-well plates, in which were added cell lines, were put in a humiditied atmosphere of 5% CO_2 at 37°C. Culture medium was discarded and cells were washed two times with warm serum-free RPMI1640. The oligonucleotides and 10ul Lipofectin were dissolved in 100ul and 90ul serum-free RPMI1640, respectively. They were mixed gently after 30 minutes in room temperature, and then the complexes were put onto the cells after diluted with 800ul serum-free RPMI1640 without antibiotics, incubated in a incubator filled with 5% CO_2 at 37°C for 6 hours. During training process, mortality rate was detected by trypan bule staining technique, mortality rate was under 5 percent. Total RNA was isolated from tranfected cell lines A-549 with TRlzol reagent (from Gibco company) according to the manufacturer`s instructions, its purity and quantification were determined by ultraviolet spectrophotometry, its integrity was verified via 1% formaldehyde denaturing gel electrophoresis. RT-PCR was performed using One-

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step kit according to the manufacturer's instructions (from KaTaRa company).The amplified system was 50ul, andβ-actin was a control. Primers was designed and synthesized by Shanghai Bio-Engineering Company as the following: HPSE(585bp),upstream 5'-TTCGATCCCAAGAAGGAATCAAC-3',downstream 5'-GTAGTGATGCCATGTAACTGAATC- 3' β-actin (250bp) upstream 5'-CAGAGCAAGAGAGGCATCC1-3' downstream 5'-GGATAGCACAGCCTGGATAG-3'.The amplification reaction involved 30 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, and 72°C for 1 minute. PCR products were observed under ultraviolet light after 1.5% agarose gel electrophoresis, and its optical density was analyzed with Gel 1D gel image analysis system(Gel Image System Ver.3.71),the net density of electrophoretic bands was calculated by the following formula: net density= average density xband area. The ratio of HPSE band density to β-actin density can indicates the relative expression of HPSE mRNA.

Rabbit anti-human HPSE monoclonal antibody working solution 100ul was added to cell suspension (1×106/ml),and incubated for 30 min at room temperature, washed with 10ml PBS. Fluorochrom-conjugated goat anti-rabbit secondary antibody solution was added. The expression of HPSE protein was quantified by flow cytometry and multicycle AV software. Fluorescence indices could indicate HPSE protein content.

24-well matrix gel invasion chambers(from BD company) were used. The size of membrane pore between upper and lower chamber is 8um, extracellular matrix gel was covered onto the upper chamber membrane surface, which can simulate the body extracellular matrix and basement membrane environment. The transfected A-549 cells were mixed with serum-free RPMI1640 to the concentration of 2×105/ml, 200ul of the cell suspension was added to the upper chamber, and 500ul RPMI1640 with 10% fetal calf serum to the lower chamber. After 48 hours in a incubator filled with 5% CO2 at 37°C, the cells into the lower chamber were fixed with formaldehyde (HE staining), and observed under the light microscope (400 times). We counted the cells in six fields randomly, mean was the number of the invasive cells. Cell invasion inhibition rate=(cell number in per field in LIP group - cell number in per field in different concentrations in ASODN group)/ cell number in per field in LIP group×100%.

Statistical Methods

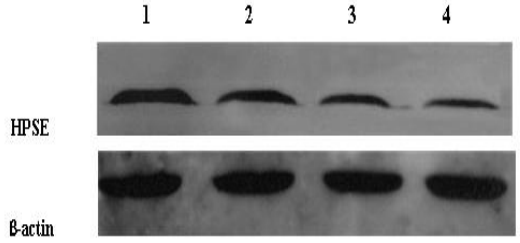
The results were expressed as mean ± standard deviation. Analysis of variance and q test were made with SPSS version 12.0 software. $p < 0.05$ was considered statistically significant.

Results

The average transfection rate of HPSE ASODN at a final concentration of 100,200, and 400nmol/L was 97%, 98%, 95%, respectively. There was no significant difference in three final concentration (p>0.05). Semi-quantitative RT-PCR amplification products electrophoresis showed that there was no significant difference in expression of HPSE mRNA between control group and LIP group (p>0.05), HPSE mRNA expression

Table 1. HPSE protein expression and invasive ability in different group (n=6, Mean±SD)

group	Fluorescence index	HPSE protein	Number of invasive cells
Control	1.00±0.00	0.62±0.03	58.5±1.22
LIP	0.92±0.02	0.65±0.03	56.3±1.35
ASODN 100	0.81±0.02	0.29±0.02	23.5±0.82
ASODN 200	0.73±0.01	0.17±0.02	12.5±0.75
ASODN 400	0.39±0.01	0.08±0.01	4.3±0.55



1.control group 2.LIP group 3.ASODN group (100 nmol/L) 4.ASODN group (400 nmol/L)

Figure 1. HPSE mRNA Expression by RT-PCR

was significantly lower in ASODN group than the other two groups (P<0.01) , and with ASODN concentration increased, HPSE mRNA expression followed by decline (Figure 1, Table 1). Flow cytometry results showed that these was no statistically significant in fluorescence index of HPSE protein expression between control group and LIP group (p>0.05). However, Lower expression of HPSE protein was characterized in ASOND groups (P<0.01), with ASODN concentration increases, HPSE protein expression followed by reduced fluorescence index and showed a dose-dependent (Figure 1, Table 1). Matrigel invasion assay showed that these was no significant in number of invasive cells between control group and LIP group (p>0.05). With increasing concentrations of

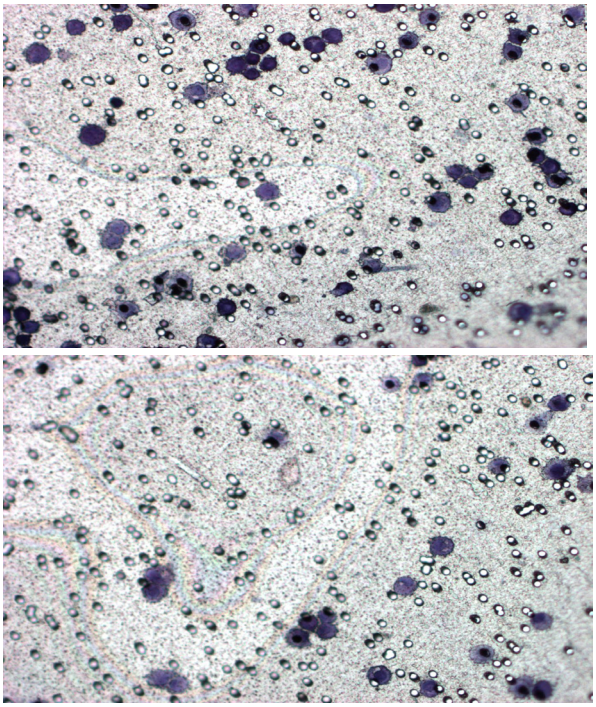


Figure 2. Numbers of A-549 Cells Invading through the Basement Membrane. HPSE ASODN Concentrations of 100 nmol/L (upper) and 400 nmol/L (lower)

ASODN, the number of invasive cells reduced. Difference was statistically significant between ASODN group and control and LIP group, the same between ASODN groups(P<0.01) (Table 1). ASODN inhibition of cell invasiveness was dose dependencies, and the inhibition rate were 55.6%, 82.3%, 91.2% at a final concentration of 100, 200, 400 nmol/L (Figure 2).

Discussion

AHPSE is an endo-β-D-glucuronidase (Nakajima and Irimura, 1998) involved in the degradation of cell surface (extracellular matrix and Heparan sulfate proteoglycans) of a wide range of normal and neoplastic tissues (Zetser et al.,2003) and a molecular determinant of metastatic events. Heparan sulfate proteoglycans are essential and ubiquitous macromolecules associated with the cell surface and extracellular matrix of a wide range of cells and tissues. HPSE is an extracellular matrix degradative enzyme, which degrades the heparan sulfate (HS) chains of Heparan sulfate proteoglycans at specific intrachain sites.The enzymatic activity of HPSE is characterized by specific intrachain heparan sulfate cleavage of glycosidic bonds with a hydrolase (but not eliminase) type of action, therefore facilitating the release of several protein modulators of cell function, including migration, adhesion, inflammation, angiogenesis, embryogenesis, and metastatic invasion (Sasaki et al., 2004). Many literatures have shown that there was high expression of HPSE in patients with cervical cancer, bone carcinoma, oral squamous cell carcinoma, hepatocellular carcinoma, breast cancer and pancreatic cancer. The most important was that the HPSE expression and patients survival time was negatively correlated (Koliopanos et a1., 2001; Maxhimer et a1., 2002; Kurokawa et a1., 2003; Shinyo et a1., 2003; Xiao et a1., 2003; Sanderson et a1., 2004). Cancer metastasis is a frequent manifestation of lung carcinoma progression. Inhibition of tumor invasion is an attractive approach for the treatment of highly malignant tumors from Zetter (1998) and Folkman (2001). So HPSE is considered as an important indicator which results in invasion and metastasis of many malignant tumor.

With complementary bases principle, ASODN can bind with the specific target mRNA sequence into the formation of DNA-RNA hybrid duplexes, which induce hydrolysis of mRNA chain by endogenous RNA enzyme H, and block shear processing and transport of mRNA and protein translation and synthesis, so as to achieve the purpose of gene therapy from Crooke (2000). Uno et al. (2001) have found the low level of HPSE expression and invasive ability of human esophageal carcinoma cell lines after transfected with HPSE ASODN. We designed and synthyzed ASODN according to HPSE mRNA start codon region, and enhance its stability and resistance to enzymatic capacity by phosphorothioate-modified. Oligonucleotides got into the cells by passive diffusion and cell membrane receptor-mediated endocytosis. Cationic liposomes could neutralize the negative charge of oligonucleotides within a certain percentage, make it easy to pass through cell membranes and improve transfection efficiency. In the present study, we have demonstrated that low expression of HPSE protein and mRNA in vitro after successful transfection, and this invasive ability was significantly reduced by inhibiting HPSE expression using liposome-mediated ASODN gene delivery strategy. In ASOND group, lower expression of HPSE and lower invasive ability to recombinate basal membrane were characterized, which were significantly different compared to the other two groups (P<0.01). However, with ASODN concentration increased, HPSE mRNA expression and protein expression followed by decline.

In conclusion, HPSE involved in lung cancer cell invasion and migration and played an important role . These observations led us to hypothesize that inhibition of HPSE expression could inhibit tumor cell invasion. HPSE may become a new target gene for lung cancer therapy, and HPSE ASODN contributes to the prevention of lung cancer cell invasion and metastasis.However, Much work remains to be performed before HPSE ASODN can be applied to treatment of human lung carcinoma.

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