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• LIVER CANCER •

Study on relationship between expression level and molecular conformations of gene drugs targeting to hepatoma cells *in vitro*

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Abstract

AIM: To increase exogenous gene expression level by modulating molecular conformations of targeting gene drugs.

METHODS: The full length cDNAs of both P_{40} and P_{35} subunits of human interleukin 12 were amplified through polymerase chain reaction (PCR) and cloned into eukaryotic expressing vectors pcDNA3.1(±) to construct plasmids of P(+)/IL-12, P(+)/P_{40} and P(-)/P_{35}. These plasmids were combined with ASOR-PLL to form two targeting gene drugs [ASOR-PLL-P(+)/IL-12 and ASOR-PLL-P(+)/P_{40} + ASOR-PLL-P(-)/P_{35}] in optimal ratios. The conformations of these two drugs at various concentrations adjuvant were examined under electron microscope (EM) and the drugs were transfected into HepG2 (ASGr+) cells. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed with total RNA extracted from the transfected cells to determine the hIL12 mRNA transcript level. The hIL12 protein in the cultured supernatant was measured with enzyme-linked immunosorbent assay (ELISA) 48 hours after transfection.

RESULTS: Targeting gene drugs, whose structures were granular and circle-like and diameters ranged from 25 nm to 150 nm, had the highest hIL-12 expression level. The hIL-12 expression level in the group co-transfected with ASOR-PLL-P(+)/P_{40} and ASOR-PLL-P(-)/P_{35} was higher than that of ASOR-PLL-P(+)/IL-12 transfected group.

CONCLUSION: The molecular conformations of targeting gene drugs play an important role in exogenous gene expression level, the best structures are granular and circle-like and their diameters range from 25 nm to 150 nm. The sizes and linking styles of exogenous genes also have some effects on their expression level.

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INTRODUCTION

In hepatoma gene therapy, the technical difficulty is that exogenous gene expression level is too low to achieve therapeutic effecets on in target cells[1]. Studies have been focused on improving gene therapeutic vector, restraining the lysosome activity in target cells, increasing exogenous gene outputs from endosomal vesicles, and modifying their transcription elements[2-7]. Whether there are some relationships between molecular conformation of gene drugs and exogenous gene expression level is not clear. Asialoorosomucoid (ASOR), the specific ligand of asialoglycoprotein receptor on the surface of hepatocytes, can be combined covalently with poly-l-lysine (PLL) to form a soluble DNA transfection vector[8-10]. Human interleukin 12 (hIL-12), also named as cytotoxin lymphocyte maturation factor or natural killer cell stimulatory factor, plays a key regulatory role in humoral immune reactions and explicits bioactivities of antivirus, antitumor and antimetastasis[11-14]. After constructing a hIL12 (human interleukin 12) double-subunit co-expressing plasmid and single-subunit gene expression plasmids, we linked them with ASOR-PLL through electrostatic interactions to form two targeting gene drugs-ASOR-PLL-DNA complexes, then examined their molecular shapes and sizes at various concentration of adjuvant under transmission electron microscope. We compared exogenous gene expression levels to select the best molecular conformation of the complexes 48 hours after transfecting them into HepG2 (ASGr+) cells, and found the means to improve the expression efficiency in target cells on the basis of molecular conformations of gene drugs.

MATERIALS AND METHODS

Materials

Eukaryotic expressing plasmids [pcDNA3.1(+/-)] and host bacteria *E. coli* JM109 were obtained from the National Laboratory of Medical Genetics of China. Plasmids purification kit 2500 was purchased from Qiagen (Chatsworth CA). All the restriction endonuclease enzymes were purchased from New England Biolabs (Beverly MA). Minimum essential medium (MEM), Trizol and lipofectamine were purchased from GibcoBRL (Grand Island NY). RT-PCR kit was purchased from Promega (Madison WI). hIL-12 (P70) ELISA kit was purchased from Pharmingen (San Diego CA). Hepatoma cells (HepG2) were obtained from China Center of Type Culture Collection (CCTCC). Newborn bovine serum was purchased from Sijiqing (Hangzhou, China). Chloroquine was a gift from Shanghai Zhong-Xi Pharmaceutical Company. All other reagents were of analytical grade from various companies in China. ASOR-PLL was prepared by ourselves.

Methods

Construction of double-subunit co-expressing plasmid of human interleukin12 The full length cDNAs of P_{40} and P_{35} subunits were amplified from human embryonic kidney by RT-PCR based on sequences deposited in Genbank (P_{40} accession number AF180563, P_{35} accession number AF180562), and cloned into pcDNA3.1(+/-) to obtain P(+)/P_{40} and P(-)/P_{35}

plasmids. The segments-P_{35}-polyA and -cmv-P_{40}-were amplified from P(-)/P_{35} and P(+)/$_{P40}$ by using the following primers IL40F, IL40R, IL35F, IL35R. They were then ligated and cloned into pcDNA3.1(+) to obtain double-subunit co-expressing plasmid P(+)/IL-12 (Table 1).

Table 1 Primers for amplifying human interleukin 12

P_{40} subunit primary PCR primers:
up stream (6044) 5'-atg tgt cac cag cag ttg gtc atc-3'
down stream (6045) 5'-gga tca gaa cct aac tgc agg gca c-3'
P40 subunit secondary PCR primers:
up stream (3479) 5'-aag gta ccg caa gat gtg tca cca gca g-3'
Kpn I
down stream (3480) 5'-gac tcg agc tgg atc aga acc taa ctg c-3'
Xho I
P_{35} subunit PCR primers:
up stream (3477) 5'-aag cta gca atg tgg ccc cct ggg tca g –3'
Nhe I
down stream (3478) 5'-ggg gta cct ttt agg aag cat tca gat g-3'
Kpn I
-cmv-P_{40}- segment PCR primers:
up stream (IL 40F) 5'-atg cat gga ggt cgc tga gta gtc –3'
Nsi I
down stream (IL40R) 5'-c atg cat cct agg tag aag gca cag tcg agg ct-3'
Nsi I Avr II
-P_{35}-polyA- segment PCR primers:
up stream (IL35-F) 5'-a cct agg cta gag aac cca ctg ctt ac-3'
Avr II
down stream (IL35R) 5'-tac ccc cta gag ccc cag-3'

Observation under transmission electron microscope After the three plasmids were mixed and incubated with ASOR-PLL at various molecular weight ratios for 40 minutes at room temperature, the mixture was electrophoresed in a 0.8 % agarose gel retardation system to determine its optimal ratio. ASOR-PLL-P(+)/P_{40}, ASOR-PLL-P(-)/P_{35} and ASOR-PLL-P (+)/IL-12 formed in optimal ratios were the two targeting gene drugs. These two drugs (plasmid DNA 0.9 μg) were formed at various concentrations of adjuvant (0, 0.1 M, 0.2 M, 0.3 M, 0.4 M, 1.5 M), then their A_{260} values were measurated and examined under a transmission electron microscope after contrast staining by 2 % uranyl acetate (*bar*=100 nm).

Cell culture and transfection HepG2 cells were cultured to 2×10^7 in minimum essential media plus 15 % fetal bovine serum (37 ℃, 5 % CO_2), and then subcultured in 12-well plates at 1:3 ratio. After the cells were cultured to 60-70 % confluence, the 6 molecule conformations of ASOR-PLL-P(+)/IL-12 and the combined ASOR-PLL-P(+)/P_{40} and ASOR-PLL-P(-)/P_{35} were transfected into the cells using liposome transfection as positive control. There were three wells in each group and the total transfected plasmid DNA was 4.5 μg for each well. During transfection, chloroquine was used (the final concentration was 100 μM*)* to restrain lysosome activity[15,16].

Detection of hIL12 expression Total RNA of transfected HepG2 was extracted by using Trizol 48 hours after transfection. mRNA expression of P_{40} and P_{35} was determined with semi-quantitative RT-PCR using β-actin as an internal control. hIL12 protein in the supernatant was measured using ELISA.

RESULTS

Construction of P(+)P_{40}, P(-)P_{35} and P(+)IL12 plasmids

The full length cDNAs of P_{40} and P_{35} subunits were amplified from human embryonic kidney and cloned into pcDNA3.1 (+/-). P (+)/P_{40} plasmids (6.4 kb) could be cut by KpnI and Xho I to produce two bands: 5.4 kb and 1.007 kb while P (-)/P_{35} plasmids could be cut Kpn I and Nhe I to produce 5.4 kb and 823 bp bands (Figure 1). The segments of -P_{35}-polyA and -cmv-P_{40}- were ligated and cloned into pcDNA3.1 (+) to get P(+)/IL-12 (8.51 kb). P(+)/IL-12 could be cut by Nhe I and Not I (2.5 kb and 6 kb) or by BamH I (2 kb and 6.5 kb) or linearized by Avr II (8.51 kb) (Figure 2).

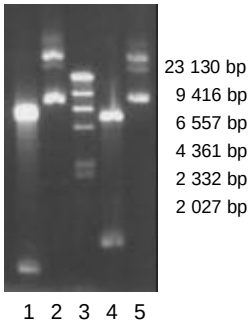

Figure 1 Electrophoresis of P(-)/P_{35}, P(+)/P_{40} and their bands after restrictive endonuclease enzyme digestion in a 0.8 % agarose gel. 1. P(-)/P_{35} plasmid digested by Kpn I and Nhe I. 2. P (-)/P_{35} plasmid. 3. Marker (λDNA/Hind III). 4. P(+)/P_{40} plasmid. 5. of P(+)/P_{40} plasmid cut by Kpn I and Xho I.

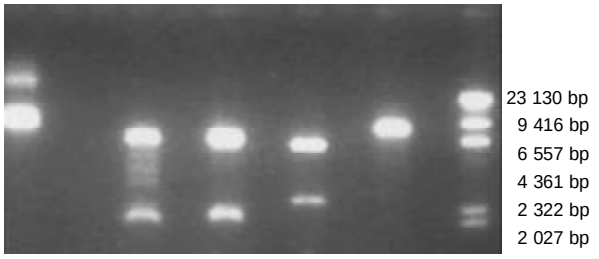

Figure 2 Electrophoresis of recombinant expression plasmid P(+)/IL-12 and the bands after restriction enzyme digestion in a 0.8 % agarose gel (Marker: λDNA/Hind III).

Molecular conformations of targeting gene drugs at various concentrations of adjuvant under transmission electron microscope

The optimal ratios of ASOR-PLL: DNA were as follow: ASOR-PLL:P(+)/IL-12=4:1, ASOR-PLL:P (+)/P_{40}[P(-)/P_{35}]=2:1. ASOR-PLL-DNA complexes which formed in 0, 0.1 M, 0.2 M, 0.3 M, 0.4 M, 1.5 M adjuvant were examined under a transmission electron microscope. The molecular conformations of gene drugs showed divarication-like (ψ-DNA) without adjuvant or in 0.3 M adjuvant, the drug molecules were condensed to granules or "doughnut"-like structures with diameters of 25 nm-300 nm in 0.1 M, 0.2 M and 0.4 M adjuvant. Their conformations varied greatly in 1.5 M adjuvant. The structure of ASOR-PLL-P(+)/IL-12 was rod-like or granule-like. The structures of ASOR-PLL-P(+)/P_{40} and ASOR-PLL-P(-)/P_{35} were both divarication-like (Figure 3).

Detection of hIL-12 expression 48 hours after drugs with different molecular conformations were transfected into HepG2 cells

Total RNA was extracted and semi-quantitative RT-PCR was performed using β-actin as an internal control. hIL12 mRNA levels were increased in both liposome-transfected and ASOR-PLL transfected groups. mRNA levels in ASOR-PLL groups varied with different concentrations of adjuvant (data not shown).

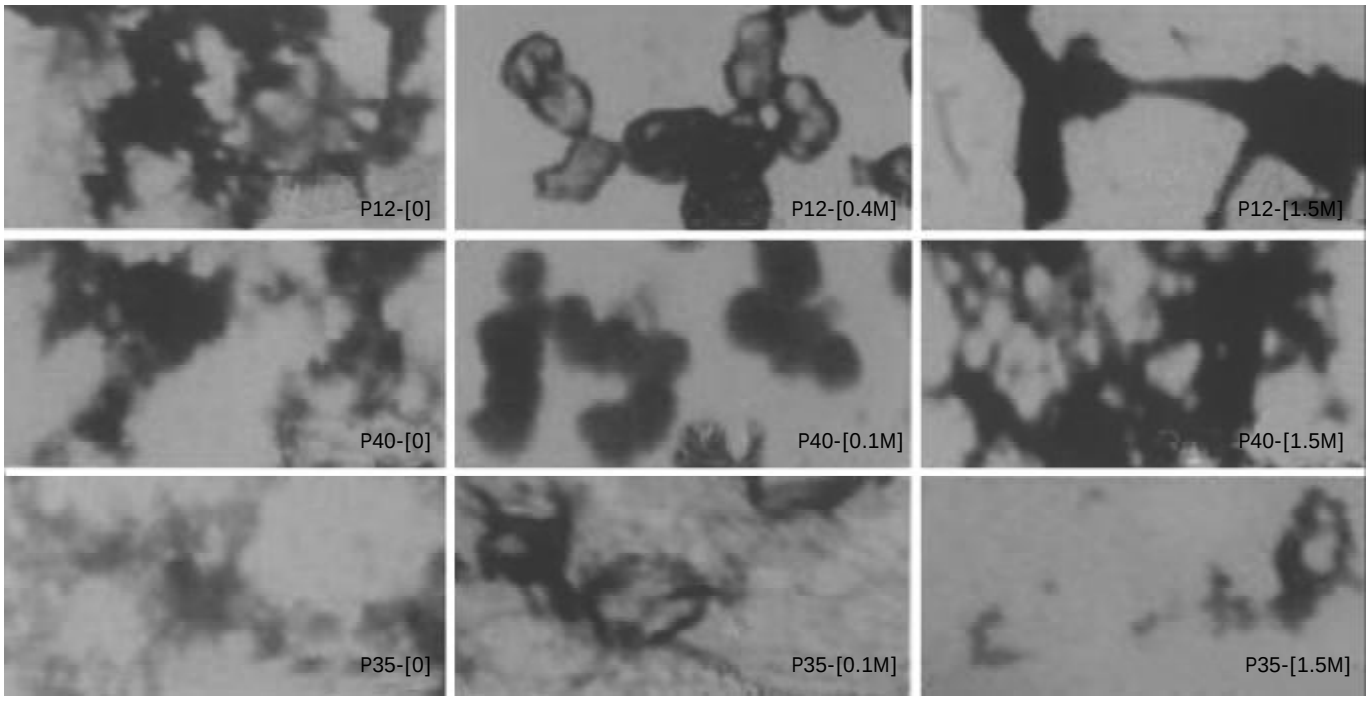

Figure 3 Differently structural features of targeting gene drugs (ASOR-PLL-DNA complexes) at various concentrations of adjuvant under transmission electron microscope. (The *bar* equals 100 nm, amplified 40 000 times).

ELISA results showed that the highest expression level of hIL12 was achieved in cells transfected with ASOR-PLL-P(+)/IL-12 in 0.4 M adjuvant, while the same level of expression was achieved when ASOR-PLL-P(+)/P_{40} and ASOR-PLL-P(-)/P_{35} were co-transfected into HepG2 cells in 0.1 M adjuvant. The expression levels in both groups went down to the lowest points in 1.5 M adjuvant. The expression levels in ASOR-PLL-P(+)/P_{40} and ASOR-PLL-P(-)/P_{35} co-transfected group were higher than those in ASOR-PLL-P(+)/IL-12 group at the same concentrations (Figure 4).

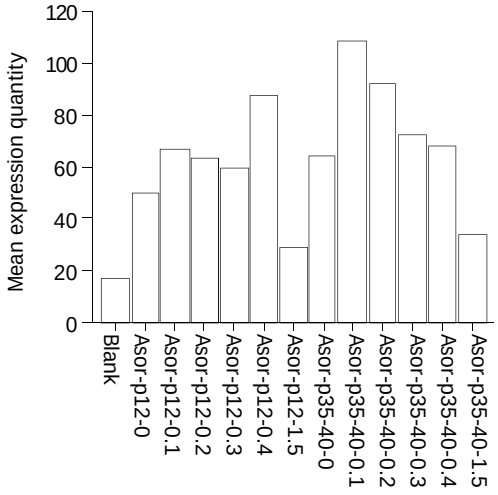

Figure 4 ELISA results of hIL-12 expressed in cell supernatant 48 hours after targeting gene drugs at various adjuvant concentrations were transfected into HepG2.

DISCUSSION

Gene transfection system mediated by receptor-ligand binding, which possesses some advantages for gene therapy, including transferring foreign genes to target cells specifically, being safer without mutation and infection caused by viral vectors, expressing functional proteins without integrating foreign genes into genomic DNA, and unlimited foreign gene size theoretically[17-19], is a potential gene therapy vector which can substitute viral vectors. ASOR is the ligand of ASG receptor (ASGr) which locates specifically on the surface of hepatocytes, and can bind to ASGr specifically[20,21]. Through the positively charged linking molecule PLL which binds to negatively charged DNA molecules through electrostatic interactions, ASOR can transfer foreign DNA molecules into hepatocytes or some hepatoma cells (ASGr+) and express the functional proteins. In 1987, Wu GY and Wu CH firstly transfected reporter gene chloramphenicol acetyltransfectase (CAT) to hepatoma cells (HepG2) by using ASOR-PLL[8]. Ever since, ASOR-PLL has been applied to various therapeutic genes targeting for hepatocytes or some hepatoma cells *in vivo* or *in vitro*[17,22,23]. However, the low expression efficiency of exogenous genes restricts its application to clinic use.

hIL-12 is a double-subunit cytokine discovered in recent years[24], which can promote Th0 cells to differentiate Th1 cells to enhance cell immunity[25]. It can also activate T cells and NK cells to proliferate and secrete IFN-γ to improve humoral immunity[26,27]. Therefore, it plays a role in antivirus, antiprimary tumors and antimetastasis[11-14]. Till now ASOR-PLL has not been used in transferring human interleukin 12 gene to HepG2. We constructed hIL12 double-subunit co-expressing plasmid P(+)/IL12 and single-subunit gene expressing plasmids P(+)/P_{40} and P(-)/P_{35}, and bound them to ASOR-PLL at various adjuvant concentrations and measured A_{260} values of the mixtures (data not shown). ASOR-PLL-P(+)/IL-12 showed the lowest A_{260} value in 0.4 M adjuvant which indicated the least dissociated DNA molecules and the highest hIL-12 expression level, while ASOR-PLL-P(+)/P_{40} and ASOR-PLL-P(-)/P_{35} showed the same result in 0.1 M adjuvant. Though ASOR-PLL-DNA complexes showed lower A_{260} values in 1.5 M adjuvant, most of the HepG2 cells could not stand such a high concentration of adjuvant and died 4 hours after they were transfected, which explained why the expression level of hIL-12 was low. These results indicate that foreign genes (hIL12) can be transferred into target cells (HepG2) through ligand (ASOR) binding to receptors (ASGr), and then endocytosed by cells as reported by Huckett *et al*[28]. The less

dissociated DNA molecules existed in the mixture, more ASOR-PLL-DNA complexes were formed, the higher efficiency of expressing hIL12 was achieved.

Pearales *et al* thought that ligand-DNA complex molecules in a receptor- mediated DNA transfection system must have a relatively high DNA concentration and certain molecule conformations to assist DNA to be transfected into target cells and express functional proteins[17]. To achieve this goal, DNA molecules need to be condensed to a smaller size, and the conformation of aggregated multiple ligand-DNA molecules (such as Ψ-DNA) inhibited gene expression. In our studies, shapes of the molecules under transmission electron microscope showed that ASOR-PLL-P(+)/IL-12 molecules were circle-like structures ("doughnut") with diameters of 50-100 nm in 0.4 M adjuvant, which produced the highest hIL-12 expression. ASOR-PLL-P(+)/P_{40} molecules were granular structures with diameters of 40-125 nm in 0.1 M adjuvant and ASOR-PLL-P(-)/P_{35} molecules were circle-like structures with diameters of 75-150 nm at the same concentration of adjuvant, both of them also achieved the highest hIL12 expression. Although ASOR-PLL-P(+)/P_{40} molecules formed granular structures with diameters of 25-75 nm in 0.2 M adjuvant, ASOR-PLL-P(-)/P_{35} molecules were concentrated to form lumps with diameters of 100-200 nm at this concentration of adjuvant. Since ASOR-PLL-P(+)/P_{40} and ASOR-PLL-P(-)/P_{35} needed to be co-transfected to express functional hIL-12, there were more factors affecting hIL-12 expression in this situation. Meanwhile, the size and the GC/AT ratio of foreign DNA molecules also affected exogenous gene expression[17]. In our studies, the smaller molecular diameters of ASOR-PLL-DNA complexes (25-150 nm), the more condensed DNA molecules and the tighter combination of DNA molecules and ASOR-PLL could lead to higher hIL-12 expression.

In general, single-subunit gene plasmids [P(+)/P_{40} and P(-)/P_{35}] co-expression mediated by ASOR-PLL could induce higher hIL12 expression than that of double-subunit genes co-expressing plasmid transfection. The possible reasons might be the following: (1) There was a certain amount of ASGr on the surface of target cells, when the same amount of DNA was used to transfect cells, ASOR-PLL-P(+)/P_{40} and ASOR-PLL-P(-)/P_{35} were co-transfected at 1:1 ratio, and ASOR could bind to ASGr more thoroughly with a relatively larger amount of ASOR, compared with ASOR-PLL-P(+)/IL-12 transfection. (2) Since the P_{40} and P_{35} subunits cDNAs were cloned in tandem into the polycloning site of pcDNA3.1(+) in which P_{40} cDNA located directly next to P_{35} cDNA, they might interfere reciprocally during transcription, and could not produce P_{40} and P_{35} subunits proportionally. However, P_{40} and P_{35} subunits must combine at 1:1 ratio to form functional hIL-12[12,25].

Another study[29], used atomic force microscope to examine DNA molecules binding to PL, ASOR-PLL and orosomucoid, and found that the optimal conformations of DNA complex molecules for foreign genes expression were solenoid-like or rod-like in diameters of 300 nm-400 nm. The condition to obtain these conformations was that ASOR was combined covalently to 10 kDa PL (Lys: nt$\geqslant$5:1), and linked to DNA molecules. In our studies, PLL was a mixture of 26 kDa, 10 kDa and 4 kDa PLL. The ratio of 10 kDa PL: nucleotide was less than 5:1 in our targeting gene drugs, so hIL-12 expression in ASOR-PLL transfection group was lower than that in liposome transfection group (data not shown).

In summary, the gene transfection system mediated by receptor-ligand binding is a prospective gene therapy strategy, and the expression level of foreign genes has a relationship with the amount of binding DNA, conformation of the complex and characteristics of the linking molecules PLL. Since hIL-12 consists of two subunits (P_{40} and P_{35}), more factors are involved in hIL-12 expression, including the way P_{40} and P_{35} genes enter into the target cells, conformations of the two ASOR-PLL-DNA complexes and interactions of the two complexes. Our studies may contribute to improving hIL-12 expression and applying hIL-12 to clinical hepatoma gene therapy research.

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