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Preparation of liposome-coated oligonucleotide labeled with ^{99m}Tc and its uptake in vascular smooth muscle cells

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Abstract To explore the preparation method of liposome-coated ^{99m}Tc-labeled antisense oligonucleotide (ASON), targeteing the proliferating cell nuclear antigen (PCNA), and to explore the biological characteristics and the uptake kinetics of a radiolabeled probe in vascular smooth muscle cells, an 18-base single-stranded antisense oligonucleotide targeting PCNA mRNA and the complementary strand (sense oligonucleotide, SON) were synthesized. The ASON (SON) was labeled with ^{99m}Tc, by conjugating the bifunctional chelator (hydrazino nicotinamide, HYNIC), and purified through a gel filtration column of Sephadex G-25. The product was then encapsulated in cationic liposome (oligofectamineTM). The radiolabeling efficiency, radiochemical purity, stability of the liposome-coated ^{99m}Tc-HYNIC-ASON in a phosphate buffered solution (PBS), and fresh human serum and its uptake rate were studied. There was no significant difference between the ^{99m}Tc radiolabeling efficiencies of HYNIC-ASON and HYNIC-SON, which were 60.04% ± 1.92% and 59.60% ± 2.53%, respectively ($P > 0.05$, $n = 5$). The radiochemical purity of the liposome-coated ^{99m}Tc-HYNIC-ASON was 94.70% ± 1.90% ($n = 5$). And after incubation with PBS and fresh human serum at a concentration of 1.2 μg·mL^{-1} for 120 min, the radiochemical purities were 92.27% and 91.55% respectively. At 90 min after transfection, the uptake rate of the liposome-coated ^{99m}Tc-HYNIC-ASON reached its peak of 83.8% ± 5.92% in vascular smooth muscle cells (VSMCs) and was much higher than that of the nonliposome-coated ^{99m}Tc-HYNIC-ASON, which was 11.16% ± 0.54% ($P < 0.01$, $n = 4$). The labeling method of PCNA ASON (SON) conjugated by HYNIC has been proved successful. The liposome was able to enhance the ASON (SON) uptake in VSMCs, and could be widely used as a safe, convenient, effective gene transfer carrier.

Keywords Proliferating cell nuclear antigen, Oligonucleotides, Antisense, Isotope labeling, Liposome, Vascular smooth muscle cells

CLC numbers R730.44, R817.4

1 Introduction

The proliferating cell nuclear antigen (PCNA) is a 36 kD polypeptide found in the nuclei of proliferating cells. PCNA functions as a cofactor of endonuclear deoxyribonucleic acid polymerase to assist DNA transactions and participates in different pathways of DNA replication and repair synthesis. Its expression effectively represents cell proliferation, and plays an important role in tumor and vascular proliferating diseases[1-4]. Accordingly, in the present study, an 18-base antisense oligonucleotide targeted at PCNA mRNA and sense oligonucleotide have been synthesized and radiolabeled with ^{99m}Tc conjugating hydrazino nicotinamide (HYNIC), and then encapsulated in oligofectamine^{TM}. The biological characteristics of this radiolabeled composite and its uptake kinetics in vascular smooth muscle cells have been studied.

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2 Materials and methods

2.1 Materials

2.1.1 Reagents

PCNA, ASON, and SON were synthesized and linked with a primary amine on the 5′ end (Beijing SBS Genetech Co.), the base sequences were 5′-GAT CAG GCG TGC CTC AAA-3′ (ASON) and 5′-TTT GAG GCA CGC CTG ATC-3′ (SON); HYNIC (synthesized according to Ref.[5] and identified by magnetic resonance and mass spectrum); Oligofectamine^{TM} and OPTI-MEMI medium (Invitrogen Co. USA); Sep-Pak C_{18} reverse-phase column (Waters Milfort M. A. USA); Gel filtration column of Sephadex G-25 (Pharmacia Co. USA); $^{99m}TcO_4^-$ (China Institute of Atomic Energy); Dimethylformamide (DMF), stannous chloride ($SnCl_2$•H_2O), and Tricine (Sigma Co. USA).

2.1.2 Animals

Male Sprague-Dawley rats weighing 200 g to 250 g, supplied by Animal Laboratory Center of Tongji Medical College of Huazhong University of Science and Technology, were used.

2.1.3 Preparation of Sep-Pak C_{18} reverse-phase column.

The Sep-Pak C_{18} column was washed with 10 mL HPLC-grade acetonitrile and rinsed with 20 mL aliquot of sterile filtered water.

2.2 Methods

2.2.1 Oligonucleotide conjugation

For conjugation, the HYNIC was dissolved in dry DMF to a concentration of 10 mg/mL, and the DNA (165 μg) was dissolved in a sterile bicarbonate buffer (0.25 mol/L, pH 8.5) so that the final concentration was 5 μg/μL. The HYNIC was added dropwise to the DNA solution, with oscillation, at a molar ratio of 20:1 (HYNIC:DNA). The solution was incubated at room temperature for 60 min, and then diluted to 1 mL and loaded onto a 4 × 25 mm Sep-Pak C_{18} reverse-phase column. The column was washed with the following solutions: 10 mL of 25 mmol/L ammonium bicarbonate (pH 8.5), 10 mL of 25 mmol/L ammonium bicarbonate/5% acetonitrile, and 2 × 10 mL water/5% acetonitrile. HYNIC-ASON (SON) was then eluted with 4 × 1 mL of water/30% acetonitrile. The absorbance of the eluted sample at 260 nm was measured using an ultraviolet spectrophotometer. The peak fractions were pooled and dispensed to a 10 μg/vial, and stored at -20℃ for future use.

2.2.2 Labeling of HYNIC-DNA

10 μg HYNIC-DNA was dissolved in 50 μL of 0.25 mol/L bicarbonate buffer (pH 8.5). To this solution were added 100 μL of 70 mg/mL tricine solution, 10 μL fresh $SnCl_2$ solution (1 mg/mL in 0.1 mol/L HCl), and 74 MBq $^{99m}TcO_4^-$. The solution was then incubated for 60 min at room temperature, and purified on a 0.7 × 20 cm gel filtration column of Sephadex G-25 column using normal saline as an eluant. The radiocounting as well as absorbance at 260 nm of each collecting duct were quantitated to calculate the radiolabeling efficiency.

2.2.3 Encapsulation

60μL ^{99m}Tc-HYNIC-ASON or ^{99m}Tc-HYNIC-SON (20μmol/L) was diluted in 960 μL OPTI-MEMI (solution A), and incubated at room temperature for 5 min; 36 μL oligofectamine^{TM} was diluted in 144 μL OPTI-MEMI (solution B), and incubated at room temperature for 5 min. Then solution A and solution B were mixed slightly and incubated at room temperature for 15 min to 20 min.

2.2.4 Radiochemical purity

Liposome-coated ^{99m}Tc-HYNIC-ASON (SON) and nonliposome-coated ^{99m}Tc-HYNIC-ASON (SON) were analyzed with Sep-Pak C_{18} reverse-phase column using 30% acetonitrile solution (dissolved in 0.1mol/L ammonium acetate solution) as an eluant. The absorbance at 260 nm and radiocounting of each duct were quantitated to calculate the radiochemical purity.

2.2.5 Labeling stability

Liposome-coated ^{99m}Tc-HYNIC-ASON and nonliposome-coated ^{99m}Tc-HYNIC-ASON were incubated with PBS and fresh human serum at a concentration of 1.2 μg/mL for 120 min at 37℃, respectively. The variations of radiochemical purity and stability were evaluated by Sep-Pak C_{18} column.

2.2.6 VSMC culture

Male Sprague-Dawley rats (200g-250g) were anesthetized, and VSMCs were isolated from the thoracic and abdominal portion of the rats' aorta by sterile operation. VSMCs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with

20% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. The number of cells could satisfy the needs after seven days. Then the cells were identified by immunohistochemistry staining of α-actin and cultured in DMEM with 10% fetal bovine serum and maintained as a monolayer. All types of primary and subcultured cells were incubated in 5% CO_2 in air at 37℃.

2.2.7 Cell uptake

The day before the uptake analysis, monostratal VSMCs were digested with 0.25% trypsin and 0.02% EDTA (mass ratio 1:1), and counted by using a counting plate. Then the cells were cultivated on 24-well cell culture plates that were washed with PBS before inoculation, and cultured in DMEM supplemented with 10% fetal bovine serum and without antibiotic, in 5% CO_2 at 37℃ for 24 h. At time zero, the culture medium was aspirated and the cell culture plates were washed twice with PBS. Then 200 μL of DMEM without serum and antibiotic was added to each well. 50 μL of liposome-coated ^{99m}Tc labeled probes were then added into each well and mixed slightly, with nonliposome-coated ^{99m}Tc labeled probes for comparison. The cells were incubated in 5% CO_2 at 37℃. At different time points of 30, 60, 90, 120, 180, and 240 min, the supernatants were removed from each of the four wells, and the cells were washed sequentially with PBS. And then the adherent cells were lysed by addition of 1 mL lysate (0.2 mol/L NaOH and 1% SDS) to each of the four wells. The radioactivity in the supernatants, washings, and cell pellets was determined with a γ counter to calculate the cell uptake rates.

2.2.8 Statistical analysis

All data were reported as mean ± SD, and performed with the Student's *t* test. All statistical tests were two-tailed, with a significance level of $P < 0.05$.

3 Results

3.1 Radiolabeling efficiency

There were stable radiolabeling efficiencies when the HYNIC-ASON (SON) was labeled with $^{99m}TcO_4^-$ several times. When the specific activity of $^{99m}TcO_4^-$ was 6.4 MBq/μg, there was no significant difference between the radiolabeling efficiencies of HYNIC-ASON and HYNIC-SON, which were 60.04% ± 1.92% and 59.60% ± 2.53%, respectively ($P > 0.05$, $n = 5$).

3.2 Radiochemical purity

After purification through the Sep-Pak C_{18} column, the radiochemical purities of nonliposome-coated ^{99m}Tc-HYNIC-ASON and nonliposome-coated ^{99m}Tc-HYNIC-SON were 95.43% ± 2.30% and 94.90% ± 1.85%, respectively ($n = 5$). The radiochemical purities of liposome-coated ^{99m}Tc-HYNIC-ASON and liposome-coated ^{99m}Tc-HYNIC-SON were 94.70% ± 1.90% and 94.15% ± 2.41%, respectively ($n = 5$).

3.3 Labeling stability

After 120 min incubation with PBS and fresh human serum at 37℃, the radiochemical purities of ^{99m}Tc-HYNIC-ASON were 92.54% and 91.72% respectively, and those of liposome-coated ^{99m}Tc-HYNIC-ASON were 92.27% and 91.55%, respectively. The ^{99m}Tc labeled ASON conjugating HYNIC was seen to be stable during PBS and serum incubations. There was no shift in the peak of radioactivity.

3.4 Cell uptake

The rates of cell uptake with ^{99m}Tc-HYNIC-DNA are shown in Table 1. At 37℃, the uptake rate of ^{99m}Tc-HYNIC-ASON and ^{99m}Tc-HYNIC-SON in vascular smooth muscle cells increased with time. At 90 min after transfection, the uptake rate of ^{99m}Tc-HYNIC-ASON and ^{99m}Tc-HYNIC-SON reached the peak of 11.16% ± 0.54% and 5.78% ± 0.72%, respectively, then reduced with time (Fig.1).

Table 1 shows that there was significant difference between ^{99m}Tc-HYNIC-ASON and ^{99m}Tc-HYNIC-SON ($t = 4.166 \sim 11.956$, $P < 0.01$, $n = 4$). The uptake rate of the liposome-coated ^{99m}Tc-HYNIC-ASON and liposome-coated ^{99m}Tc-HYNIC-SON at 90 min reached the peak of 83.8% ± 5.92% and 51.6% ± 1.36%, respectively, and were much higher than that of the nonliposome-coated ^{99m}Tc-HYNIC-ASON and ^{99m}Tc-HYNIC-SON (t= 24.439, 59.552, $P < 0.01$, $n = 4$). There were significant differences between the uptake rates of ^{99m}Tc-HYNIC-ASON and liposome-coated ^{99m}Tc- HYNIC-ASON, as well as those of ^{99m}Tc-HYNIC-SON and liposome-coated ^{99m}Tc-

HYNIC-SON (t= 21.521 ~ 111.021, 29.241 ~ 117.585, $P < 0.01$, $n = 4$). And the uptake rates of liposome-coated ^{99m}Tc-HYNIC-ASON were much higher than those of liposome-coated ^{99m}Tc-HYNIC- SON (t = 6.300 ~ 26.405, $P < 0.01$, $n = 4$).

Table 1 Uptake rate of liposome-coated ^{99m}Tc-HYNIC-ASON(SON) and nonliposome-coated ^{99m}Tc-HYNIC-ASON(SON) in VSMCs ($\bar{x} \pm s$, %, $n = 4$)

Agent	Time /min					
	30	60	90	120	180	240
^{99}Tc^{m}-HYNIC-ASON$^{\bigstar}$	4.09±0.76	7.40±0.58	11.16±0.54	7.42±0.35	6.19±0.16	5.81±0.82
^{99}Tc^{m}-HYNIC-SON$^{\blacklozenge}$	1.38±0.28	3.19±0.43	5.78±0.72	4.50±1.06	4.07±0.39	3.73±0.57
Oli-^{99}Tc^{m}-HYNIC-ASON$^{\blacktriangle}$	60.8±1.69	75.2±4.48	83.8±5.92	72.4±4.77	64.6±1.04	59.8±4.95
Oli-^{99}Tc^{m}- HYNIC-SON$^{\bullet}$	36.9±2.25	44.7±0.56	51.6±1.36	46.5±2.67	44.9±1.07	43.8±1.14
$t^{\bigstar\blacklozenge}$	6.692	11.662	11.956	5.232	10.058	4.166
$t^{\blacktriangle\bullet}$	16.987	13.511	10.602	9.476	26.405	6.300
$t^{\bigstar\blacktriangle}$	61.208	30.017	24.439	27.172	111.021	21.521
$t^{\blacklozenge\bullet}$	31.332	117.585	59.552	29.241	71.703	62.877

All data were obtained with the Student's t test, $P^{\bigstar\blacklozenge}$, $P^{\blacktriangle\bullet}$, $P^{\bigstar\blacktriangle}$ and $P^{\blacklozenge\bullet} < 0.01$. Oli-^{99m}Tc-HYNIC-ASON = Liposome-coated ^{99m}Tc-HYNIC-ASON. Oli-^{99m}Tc-HYNIC-SON = Liposome-coated ^{99m}Tc-HYNIC-SON.

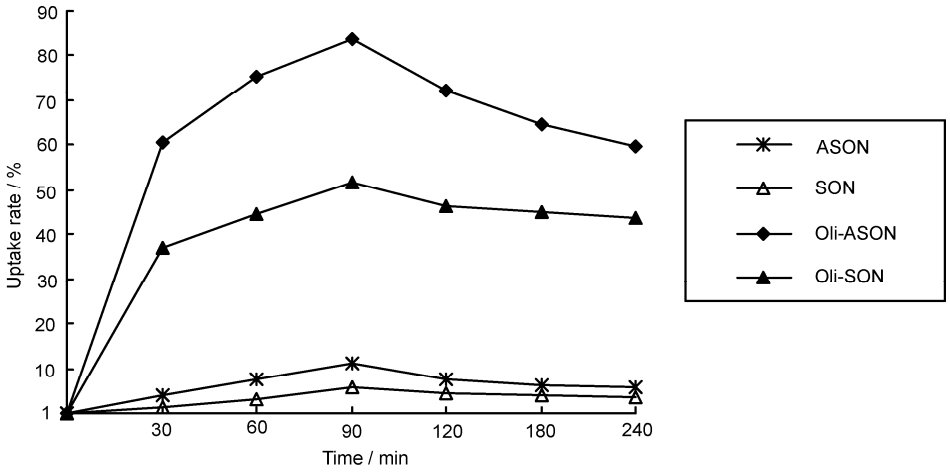

Fig.1 Cell uptake studies of VSMCs revealed that liposome-coated ^{99m}Tc-HYNIC-ASON/SON showed significantly higher accumulation than nonliposome-coated ^{99m}Tc-HYNIC-ASON/SON in VSMCs. At 90 min after transfection, the uptake rate of nonliposome-coated ^{99m}Tc-HYNIC-ASON/SON reached the peak of 11.16% ± 0.54% and 5.78% ± 0.72%, respectively. And the uptake rate of liposome-coated ^{99m}Tc-HYNIC-ASON/SON reached the peak of 83.8% ± 5.92% and 51.6% ± 1.36%, respectively.

4 Discussion

Atherosclerosis (AS) is one of the most common diseases for old people, with an incidence rate of 79.9% in Chinese people of 60 and upward. Atherosclerosis is a disease with high incidence, mortality, and recurrence, which seriously jeopardizes the health of old people. The existing researches indicate that abnormal proliferation and immigration of vascular smooth muscle cells play an important role in the generation of atherosclerosis, which are the chief causes of restenosis after the intracavitary plasty of the coronary artery.[6] PCNA acts as an auxiliary subunit of DNA polymerase δ that stimulates cells to get into the S phase from the G_0 phase, and assists the synthesis of the aiming strand and lagging strand of DNA during cell division. Researches indicate that cell division and proliferation cannot be carried out if the activity of PCNA is restrained. Oligonucleotides targeting PCNA can control the expression of the PCNA gene and inhibit the proliferation of VSMCs.[4]

The specific binding of the oligonucleotide and the target gene or mRNA of the target gene is according to base complementary pairing, and the expression

of the target gene is therefore depressed, with high selectivity and specificity.[7] Atherosclerotic plaques of rabbit models have been successfully imaged by Qing Guangming[8] using ^{99m}Tc labeled oligonucleotide targeting c-myc mRNA. In the present study, experiments have been carried out in vitro using oligonucleotide targeting PCNA mRNA, to explore a method of labeling the PCNA oligonucleotide with ^{99m}Tc, to note the uptake in vascular smooth muscle cells, and to evaluate the feasibility of further imaging applications in vivo.

DNA containing natural phosphodiester bonds especially single-stranded DNA can be rapidly decomposed by nucleinase in biological systems, which strongly restrict their applications. Accordingly, the principal chain of oligonucleotide has been derivatized with phosphorothioic acid, with a primary amine on the 5′ end, to enhance the affinity of oligonucleotide to target, and the resistance to nucleinase the exterior and interior. There is a hexyl ($(CH_2)_6$) between the framework and amino group, to which the chelator is then covalently conjugated.[9] A chelator is imperative in the method of labeling ASON with ^{99m}Tc. Among the various chelating agents developed to date, diethylenetriaminepentaacetic acid (DTPA) and its derivatives have been frequently used for labeling proteins with ^{111}In and several other radionuclides, but it is less successful with ^{99m}Tc because of the poor labeling stability. HYNIC constitutes a representative bifunctional agent for ^{99m}Tc radiolabeling and has been used in various target molecules. ASON can be conjugated swiftly and efficiently with a small dose of HYNIC at room temperature, and can be radiolabeled stably with ^{99m}Tc.[10]

Stable radiolabeling efficiencies were obtained when ASON (SON) was labeled with $^{99m}TcO_4^-$ conjugating HYNIC several times. After being purified through the Sep-Pak C_{18} column, the radiochemical purities of ^{99m}Tc-HYNIC-ASON and liposome-coated ^{99m}Tc-HYNIC-ASON were 95.43% ± 2.30% and 94.70% ± 1.90%, respectively. The stability of a label in a serum is among the most important factors for an agent under consideration for in vivo use. After incubation with PBS and fresh human serum for 120 min at 37℃, the radiochemical purities of ^{99m}Tc-HYNIC-ASON and liposome-coated ^{99m}Tc-HYNIC-ASON were above 90%. It displayed acceptable stability of the labeled compound in vitro, and further applications of experiments and clinical researches are expected. Compared to SON groups, ASON could be selectively taken up by VSMCs in the cell uptake experiments. The combination of ASON and target gene was specific according to base complementary pairing, and not influenced by chemical modification, labeling, or encapsulation with liposome.

One of the primary requirements for oligonucleotide to be successful as an antisense agent is for it to be taken up by the target cells in a reasonable quantity. It is difficult for natural oligonucleotide to efficiently cross the cell membranes and be taken up by cells and tissues, because of its large molecular weight, high hydrophilicity, and strong negative charges on the molecular surface. In this study, the peak of uptake rate of ^{99m}Tc-labeled ASON in VSMCs was only 11.16% ± 0.54% at 90 min after transfection. The oligonucleotide must, although still keeping intact, find its way into the cytoplasmic compartment where the mRNA is situated, before it is able to reach its target in sufficient concentration. Cationic liposome, as an effective carrier, can combine with negatively charged ASON by electrostatic interaction, protecting the oligonucleotide from nuclease degradation. In addition, it is easy for the cationic lipid-DNA complexes with positive charges to combine with cells with negative charges and be internalized in cells. Then the cellular uptake of ASON in VSMCs is effectively improved, but the toxicity is reduced. As reported, confocal microscopy analysis has indicated that the ingression of cationic lipid-DNA complex into the endochylema has resulted in efficient complex dissociation and biological activities.[11, 12] This report shows that the stability of the oligonucleotide was enhanced because it was encapsulated in a cationic liposome, also with a significantly increased uptake in target cells. At 90 min after transfection, the uptake rate of the liposome-coated ^{99m}Tc-HYNIC-ASON reached its peak of 83.8% ± 5.92% in VSMCs and was much higher than that of the nonliposome-coated ^{99m}Tc-HYNIC-ASON ($P < 0.01$, $n = 4$). It follows that the cationic liposome, as an effective delivery carrier for gene transfection, can enhance the cellular uptake of ASON in target cells, surmount their instability, and reach their insufficient

concentration for binding with the target gene.

5 Conclusions

Ideal radiochemical purity and stability, as well as reduced toxicity and higher transfection efficiency could be obtained after $^{99}Tc^{m}$-HYNIC-ASON was encapsulated in liposome, which is expected in further in vivo imaging applications of atherosclerotic plaque in animal models.

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