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Experimental study of triplex-forming oligonucleotide targeted to the initiator of S gene of HBV labeled with ^{125}I

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Abstract This study is used to investigate the feasibility of employing the Iodogen method to label triplex-forming oligonucleotide (TFO) targeted to the initiator of the S gene of HBV with ^{125}I. A 17-mer oligonucleotides sequence was synthesized and grafted at the 5′ terminal with a tyramine group. Radioiodination of the tyramine-TFO with ^{125}I was then performed using the Iodogen method. After TFO was labeled with ^{125}I using the Iodogen method, the labeling rate, the radiochemical purity, stability and bioactivity were determined, respectively. The results show that the radiolabeling rate and the radiochemical purity were 93% and 99%, respectively; and the radiochemical purity is more than 90% *in vitro* at -20°C on the 5th day after labeling; and the rate of ^{125}I-tyramine-TFO binding to HepG2.2.15 cells was （37.2±1.4）% and statistically different from the rate of HepG2 ($p<0.5$). Hence, it is concluded that the labeling of oligonucleotides conjugated with tyramine using the Iodogen method is successful and is characterized with a high labeling rate, high stability, and a low loss of bioactivity of the labeled agent.

Key words Triplex-forming oligonucleotide(TFO), ^{125}I, Labeling
CLC number R817

1 Introduction

Antigen radiotherapy (AR) is based on targeting localized radiodamage to specific sites in the genome using sequence-specific triplex-forming oligonucleotides (TFO) to carry Auger-electron-emitters (A-Ettr) such as iodine-125 (^{125}I) to the target gene sequence[1]. The decay of ^{125}I that releases a shower of low energy electrons that produce DNA strand breaks mostly within 10 bp from the decay site [2,3] with an efficiency close to 1 break/decay [4]. ^{125}I, delivered by a TFO, should produce double-strand breaks (dsb) localized to the specific DNA target sequence.

The Shanghai Biochemistry Institute has developed TFOs targeted to the initiator of the S gene of HBV, which can block transcription and expression of the target gene by binding to the major groove of complementary duplexes and forming triple helical structures or triplexes.[5] The use of ^{125}I-labelled TFO is proposed to deliver radionuclides to target DNA sequences so that the decay will produce sequence-specific DNA breaks. However, labeling the oligonucleotide with ^{125}I without loss of bioactivity is the key factor.

There are various methods of labeling oligonucleotides with ^{125}I. Panyutin [6] labeled a TFO with ^{125}I at the C5 position of a single deoxycytosine residue by incorporating [^{125}I] IdCTP into TFO using the primer extension method, but this method is not suitable for longer oligonucleotides[7]. Dewanjee [8] developed the techniques of radioiodination by the conjugation of oligonucleotides with p-methoxyphenyl isothiocyanate

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(PMPITC), but the yield of radioiodination was as low as 50%—60%, and the labeled compound was not stable enough because experiments on animals showed that thyroid had a high uptake of radioiodine at 1 hour after the labeled compound was injected[9]. In Cammilleri's study[10], a 23-mer oligonucleotide sequence was synthesized and grafted at the 5' terminal with a tyramine group that was further radioiodinated, which proved that the 5' tyramine group allowed specific and stable radiolabeling of the AS with ^{125}I. On this basis, we try to synthesize a TFO and graft it at the 5' terminal with a tyramine group, then label it with ^{125}I using the Iodogen method and determine the optimum conditions of radioiodination.

2 Materials and methods

2.1 Materials

2.1.1 Tyramine-TFO: synthesized by Shanghai Shenenergy Biocolor Biomedicine Limited Company;

2.1.2 ^{125}I-NaI: produced by Chengdu Gaotong Isotope Corporation (China Nuclear Group), radioactivity concentration 11.4 GBq/mL, colorless and clear liquid, pH 7.0, without reducer, radiochemical purity 99.5%, the lot number: 200-10-02;

2.1.3 FMJ-184 radioimmunity γ count meter: produced by Rihuan Instrument Factory of Shanghai;

2.1.4 Ultraviolet analytical apparatus: Shanghai Gucun Shenguang Instrument Co., Ltd.;

2.1.5 Iodogen: produced by Sigma Company;

2.1.6 Sephadex G-10: imported from USA and distributed by Shanghai Chemical Reagents Company;

2.1.7 HepG2.2.15 cells: given kindly by Prof. Wang Shengqi, Laboratory of Beijing Radiology Medicine Institute.

2.2 Methods

2.2.1 TFO synthesis

The Shanghai Biochemistry Institute has designed five TFOs, which can bind specifically to the initiator of the S gene of HBV and form triple helical structures to block the transcription and reduplication of the targeting gene. One of these was picked up with a sequence of 5′AAG GAG GAG GAG GGA GG 3′, and synthesized by the Shanghai Shenenergy Biocolor Biomedicine Limited Company. The Nuclease-resistant phosphorothioate derivatives of oligonucleotides were synthesized on an ABI-3900 DNA synthesizer followed by purification from a polyacrylamide gel (PAG). Besides, the TFO was grafted with a tyramine group at the 5'-terminal, which was further radioiodinated.

2.2.2 Labelling tyramine-TFO with ^{125}I using the Iodogen method

Iodogen was dissolved in dichloromethane at a concentration of 250 μg/mL, and 60 μL was dried to the inner walls of an Eppendorf tube under a nitrogen stream. A teflon-coated magnetic stir bar (1 mm) was placed in the EP tube. The iodogen-coated tube was placed on a magnetic stirrer under a fume hood. 33 μg of tyramine-TFO in 10 μL of PBS (0.02 mol/L, pH 7.2) was mixed with 0.2 μL (4.6 MBq) of ^{125}I and the solution was transferred to the Iodogen-coated tube with the stir bar inside. After a stirring time of 15 min at 0°C, the reaction was terminated by adding 80 μL of PBS to the tube.

To observe the labeling rates on different reaction time (RT), the RT was prolonged to 30min, 1 h, 2 h, and 4 h, whereas the other reaction conditions were fixed.

2.2.3 Separation and purification

The radioiodinated materials were chromatographed on a Sephadex G-10 (7.8 mm × 200 mm), eluted by 0.02 mol/L PBS (pH 7.2) and collected as 1-mL fractions. The first peak was ^{125}I-labeled tyramine-TFO and the second peak was free ^{125}I. The ^{125}I-tyramine-TFO was collected, its radiochemical purity was measured, and its specific activity was then calculated.

2.2.4 Measurement of the labeling rate and the radiochemical purity

The labeling rate and the radiochemical purity were measured by paper chromatography using Xinhua No.1 filter paper, and ethanol: distilled water (70:30 *V*/*V*) as the mobile phase.

2.2.5 Bioactivity of the ^{125}I-tyramine-TFO

According to this protocol, a coprecipitate of TFO and calcium phosphate was formed, which was engulfed into the cell through phagocytosis. This method was used to transfect the HepG2.2.15 cells. The cells were split from the culture bottle to 6-cm culture plates 24 h before the transfection and grown

under standard conditions. The TFO/calcium phosphate coprecipitate was prepared as follows: per 6-cm plate, 150 μL of ^{125}I-tyramine-TFO solution (10.5 kBq) was mixed with 150 μL of 0.5 mol/L $CaCl_2$ solution and after thorough mixing, 300 μL of 2 × HBS was added (slowly pipetting and simultaneous vortexing). The mixture was then incubated for 30 min at room temperature to allow the formation of the coprecipitate. After incubation, the mixture containing the coprecipitate was vortexed and pipetted into 6 cm plates (600 μL per plate). After the cells were incubated with the TFO/calcium phosphate coprecipitate in 5% CO_2, at 37 °C, in an incubator for 4 h, the cells were treated with 1 × HBS/15% glycerin for 4 min at room temperature. Then, the precipitate-containing medium was discarded and the cells were washed twice with 5 mL of 1 × PBS buffer. The cells were grown for an additional 48 h in a fresh culture medium (5 mL per plate) and were then harvested. After centrifugation, the cells were collected and measured using an FMJ-184 radioimmunity γ count meter. The rate of ^{125}I-tyramine-TFO transfected to HepG2.2.15 cells was calculated by dividing the radioactivity counts of the cells by that of ^{125}I-tyramine-TFO.

As a contrast, the ^{125}I-tyramine-TFO was similarly transfected to HepG2 cells, which did not contain HBV DNA.

3 Results and discussion

3.1 The optimum radioiodination condition

In this study, the radioiodination techniques have been used by conjugating oligonucleotides with the tyramine group and by optimizing the radioiodination conditions, such as the concentration of reactants, pH, time, and temperature.

As TFO is not stable at room temperature[11] , and the optimum pH and the temperature of the radioiodination is 7.2 and 0°C, respectively, the optimum reaction time (RT) has to be determined. The TFO was labeled for different RT at the optimum temperature and pH. The outcome is shown in Fig 1. It is found that as RT increases, the labeling rate becomes higher within 2 hours. However, the labeling rate eventually reaches a plateau and no longer increases when the RT exceeds 2 hours. The labeling rate and the radiochemical purity under the optimum condition is 93% and 99%, respectively.

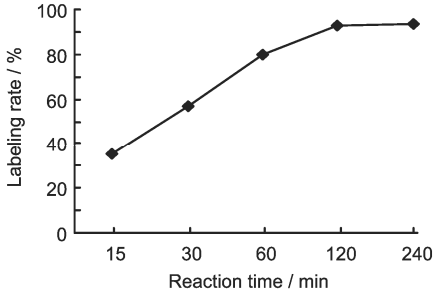

Fig.1 Labeling rate vs. reaction time.

3.2 Stability of the ^{125}I-tyramine-TFO

To test the stability of the ^{125}I-tyramine-TFO *in vitro*, its radiochemical purity was measured after the sample was stored at 37 °C and -20 °C *in vitro* for different timings. The outcome is illustrated in Figs. 2 and 3, respectively. It is seen that the radiochemical purity is more than 90% *in vitro* at -20°C on the 5th day after labeling, indicating that the ^{125}I-tyramine-TFO is considerably stable *in vitro*.

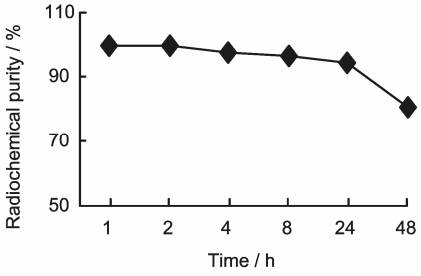

Fig.2 Radiochemical purity vs. time at 37 °C.

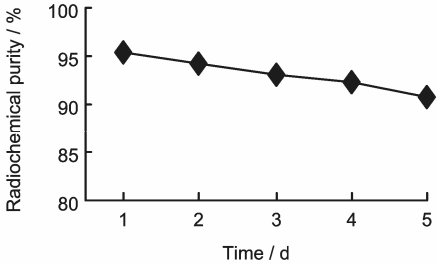

Fig.3 Radiochemical purity vs. time at -20 °C.

In addition, as to either antigen imaging or antigen therapy, oligonucleotides must pass through the cell membrane and the nuclear membrane and reach the nucleus, where these should avoid degradation by nuclease in the serum, and have enough time to bind with the target gene and exert its effect on the gene.

There are various chemical modifications of oligonucleotides [12]. In our study, the nuclease-resistant phosphorothioate derivatives of oligonucleotides have been used. Moreover, the TFO was modified by conjugation with the addition of the tyramine group at the 5'-terminal, which was further radioiodinated. All these prevent the TFO from being degraded by exonuclease, and hence the TFO is stable enough to produce its therapeutic effect. After the ^{125}I-tyramine-TFO was incubated in the fresh blood serum for 2 h, 4 h, 8 h, and 24 h, its radiochemical purity was found to become 92.2%, 85.6%, 79.8%, and 70.2%, respectively. This result suggests that the ^{125}I-tyramine-TFO could resist degradation by nuclease in the serum and be stable enough for further experiments, and thus, the performed radioiodination has been successful.

3.3 Bioactivity of the ^{125}I-tyramine-TFO

In the cell-binding experiment *in vitro*, the ^{125}I-tyramine-TFO was transducted into HepG2.2.15 cells by the calcium phosphate coprecipitate method, and the transfection rate was (37.2 ± 1.4)%. This result is close to that obtained by Lu Changde et al, who incubated the same TFO with the plasmid containing HBV DNA and got a nearly similar transfection rate[5]. On the other hand, the rate of HepG2 cells was only (7.1 ± 1.1)%, which is statistically different from the transfection rate of HepG2.2.15 cells ($p < 0.5$), which meant that the ^{125}I-tyramine-TFO designed as a complement to the polypurine/polypyrimidine region of the HBV gene could bind specially the HBV DNA in HepG2.2.15 cells. This proved that the bioactivity of TFO was not destroyed by iodination. Therefore, the ^{125}I-tyramine-TFO will be further studied in cells and animal models to show its potential therapeutic applications in posthepatitic hepatic cellular cancer (HCC).

4 Conclusion

Antigen radiotherapy (AR) is based on targeting localized radiodamage to specific sites in the genome by using sequence-specific triplex-forming oligonucleotides (TFO) to carry Auger-electron-emitters (A-Ettr) such as ^{125}I to the target gene sequence. In our study, a 17-mer oligonucleotide sequence was synthesized and grafted at the 5'-terminal with a tyramine group, which was further radioiodinated using the Iodogen method. The study proves that this method allows specific and stable radiolabeling of oligonucleotides with ^{125}I. The cell-binding experiment *in vitro* proved that ^{125}I-tyramine-TFO complementary to the polypurine/polypyrimidine region of the HBV gene can bind well with the HepG2.2.15 cell. This data will contribute to the further *in vitro* and *in vivo* experiments of antigen radiotherapy for posthepatitic HCC.

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