Preparation of ^{99m}Tc-HYNIC-Annexin V

LAN Xiao-Li, ZHANG Yong-Xue

(Department of Nuclear Medicine，Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022)

Abstract ^{99m}Tc-HYNIC-Annexin V was prepared for apoptosis radionuclide imaging in vivo. The expression plasmid of human Annexin V was constructed and expressed in *E. coli.*. HYNIC was prepared and used as bifunctional chelating agent for ^{99m}Tc labeling. The radiochemical purity, radiolabeling yield and stability of ^{99m}Tc-HYNIC-Annexin V were studied. The coding sequence of human Annexin V gene was successfully cloned into the expression vector, and highly expressed in *E. coli*. A protein with a molecular weight of about 36kD could be induced and confirmed by Western Blotting and SDS-PAGE. Annexin V could be labeled with ^{99m}Tc using HYNIC，and showed high radiochemical purity of more than 90%. The radiolabeling yield could reach more than 90%. The reaction condition was moderate. It suggested that ^{99m}Tc-HYNIC-Annexin V may be a promising agent for apoptosis imaging and clinical application.

Keywords Annexin V, Apoptosis, ^{99m}Tc labeling, HYNIC

CLC number R817

1 Introduction

Apoptosis or programmed cell death plays an important role in homeostasis and embryogenesis.[1,2] Apoptosis is an intimate component of normal organ function. On the other hand, excessive or deficient apoptosis is a major cause of diseases.[3] Although the importance of apoptosis in disease is well appreciated, laboratory tests for apoptosis are not ordered routinely for diagnosis or to plan therapy, because of the complexity of making these measurements. Annexin V, a 36kD human protein, has a high affinity for cell membranes with bound phosphatidylserine (PS).[4] Until now, fluorescein-labeled Annexin V derivatives have been widely used for in vitro visualization of apoptosis. In this study, genetic engineering was used to construct and express Annexin V in *E. coli*. ^{99m}Tc labeling was completed with bifunctional chelating agent-hydrazino nicotinamido(HYNIC). The preparation of ^{99m}Tc-HYNIC-Annexin V will allow the radionuclide imaging to detect and quantify apoptosis in vivo, and this technique will become a clinically useful determination.

2 Materials and methods

2.1 Materials

2.1.1 Reagents

pOTB7 (1815bp, vector with plasmid cDNA, from PTGLAB), expression vector pET-28a(+) (from Novagen), isopropyl-l-thiol-β-D-galactoside (IPTG, from Promega), Ni-NTA Superflow (from QIAGEN), $SnCl_2 \cdot 2H_2O$ and tricine (from Sigma), instant thin-layer chromatography strip (ITLC-SG, from Gelman Sciences), 6-chloronicotinic acid (from Acros Organics), 85% hydrazine hydrate and N,N- Dimethylformamide (DMF, from Beijing Chemical Corp.), and dicyclohexylcarbodiimide (DCC, from Jieer Biochemical Corp.) were used in the work. Other reagents were from Shanghai Chemical Corp. and all were of reagent grade.

2.1.2 Instruments

T3 Thermocycler (from Eppendorf Scientific. Inc, Germany), Electrophoresis (from Bio-RAD), Transfer Electrophoresis Equipment (from HOEFER Scientific Instruments, USA) and Nuclear Magnetic Resonance (NMR, BRUKER ARX400, 400 MHz) were used in

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the work.

2.2 Methods

2.2.1 Expression and identifying of human Annexin V

The primer was designed according to the sequence of Annexin V cDNA. The upper stream sequence was 5'-TTTT GGA TCC ATG GCA CAG GTT CTC AGA GGC, and the down stream sequence was 3'-TTTT GAA TTC TTA GTC ATC TTC TCC ACA GAG. The production of PCR was cloned into expression vector pET-28a(+), and the vector plasmid with aimed genetic sequence was transferred into *E. coli*. DH5α. The sequence of the recombined plasmid was measured by Shanghai Kairui Corp. from positive and negative directions. IPTG was used to induce expression and Ni-NTA Superflow was used to purify the protein. The purified protein was condensed by dialysis. 10% SDS-PAGE and Western Blotting were used to identify the production.

2.2.2 Preparation of HYNIC

According to the methods by Abram,[5] HYNIC was produced by 6-chloronicotinic acid with four steps; hydrazine replacement, BOC protection, NHS connection and BOC removing (Fig.1). Some purification steps were modified. For example, in the step Ⅱ and Ⅲ, purification was made by different physical characteristic instead of silica gel used in Ref.[5]. The production of every step was identified by NMR.

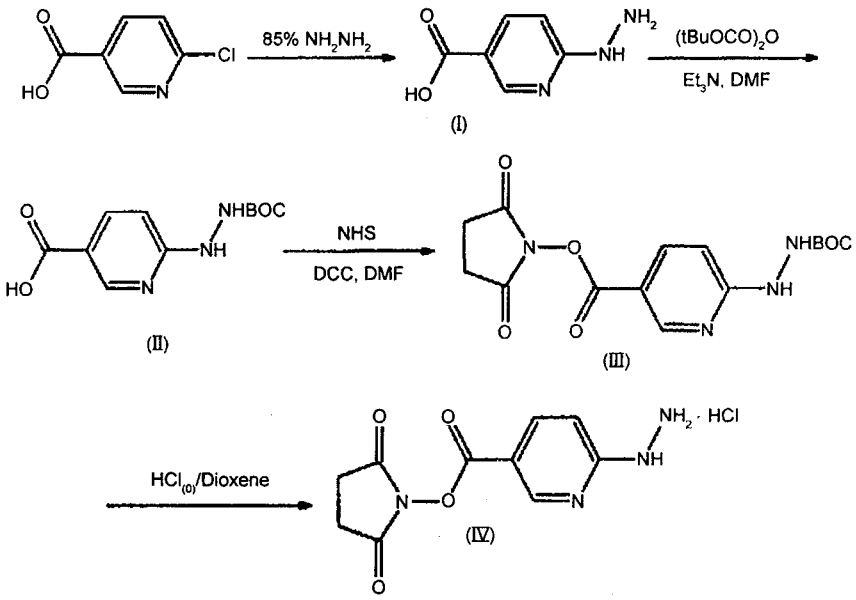

Fig.1 Steps of synthesizing HYNIC.

2.2.3 Conjugation of HYNIC-Annexin V

HYNIC was dissolved in DMF at a concentration of 42mmol/L and a 20 molar excess was added to a solution of Annexin V (3mg/mL in PBS). The reaction mixture was incubated for 3h at room temperature, protected from light. To quench the reaction, 0.5mol/L glycine solution in PBS (pH 7.4) was added and stirring for 10min. After this, the solution was dialyzed at 4℃ against a solution containing 20mmol/L citrate and 100mmol/L NaCl (pH 5.2) overnight. Precipitate was then removed by centrifuge at 15000g for 10min. Finally, the solution of HYNIC-Annexin V was divided into 100μL aliquots and stored at –20℃ until the day of use.

2.2.4 Preparation of ^{99m}Tc-HYNIC-Annexin V

Labeling with ^{99m}Tc was performed by consecutive addition of $SnCl_2 \cdot 2H_2O$ (1mg/mL in 0.05mol/L HCl), tricine solution (100mg/mL in 20mmol/L citrate buffer, pH 5.2) and $^{99m}TcO_4^-$ (74—370MBq) to a vial containing HYNIC-Annexin V. The mixture was incubated at room temperature for 1h.

2.2.5 Purification and identifying ^{99m}Tc-HYNIC-Annexin V

^{99m}Tc-HYNIC-Annexin V was purified by a Φ 0.7 cm×20cm gel filtration column of Sephadex G-25 using 0.01mol/L PBS (pH 7.4) as eluant. Radio-

activity and the 280nm absorbency were detected to identify peak fractions and count yield. The control labeling was performed using the native unconjugated protein subjected to the identical labeling procedure.

Radiochemical purity was assessed by thin-layer chromatography on ITLC-SG using acetone and saline as the mobile phase. Acetone was used for determination of the percentage of $^{99m}TcO_4^-$, and saline for the overall radiochemical purity. ^{99m}Tc-HYNIC-Annexin V was incubated in the eluant for 1h, 2h, 4h and 6h and ITLC was performed to evaluate the stability of the ^{99m}Tc-HYNIC-Annexin V.

3 Results

3.1 Expression of Annexin V

The results of measuring sequence from positive and negative directions showed that the sequence of the recombined plasmid was the same as that reported in Gene Bank and literature,[6] which suggested the success of vector construction of human Annexin V. The result of 10% SDS-PAGE showed a relatively dense protein zone between 14.4—116kD and the molecular weight was just the expected Annexin V molecular weight 36kD (Fig.2). After condensation, the content of protein could reach 3mg/mL. A clear zone between 35kD and 40kD in Western Blotting suggested that Annexin V could express stably in *E. coli* (Fig.3).

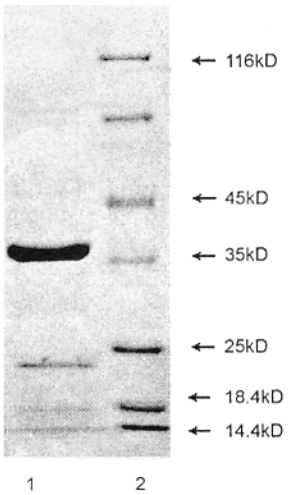

Fig.2 Result of 10%SDS-PAGE.
(1. Protein of Annexin V; 2. Marker)

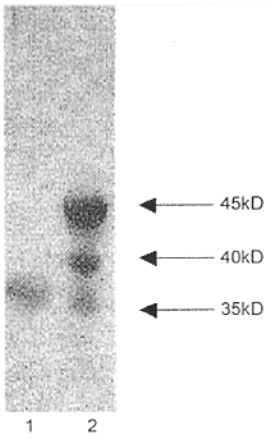

Fig.3 Result of Western Blotting.
(1. Protein of Annexin V; 2. Marker)

3.2 Production of HYNIC

According to the synthesis method of Abram,[5] some purification steps were improved. The product was proved to be HYNIC by NMR. The method is simple, the yield is steady and reasonably high, and the reaction condition is moderate. The overall yield amounted to 25%.

The NMR results in this study are: 2.89 (s 4H), 7.05 (d,j=8.8Hz, 1H), 8.22 (dd,j=2.4, 8.8Hz, 1H), 8.86 (d,j=2.4Hz, 1H); while the NMR results in Ref.[5] are: 2.88 (s 4H), 7.01 (d,j=8.8Hz, 1H), 8.19 (dd,j=2.4, 8.8Hz, 1H), 8.83 (d,j=2.4Hz, 1H). Both are very approaching.

3.3 Results of ^{99m}Tc labeling

The radiolabeling yield could reach (96.32± 2.08)% (n=6). The results of radioactivity counts and the 280nm absorbency after purification with Sephadex G25 were shown in Fig.4. The radiochemical purity was (96.90±2.27)% (n=6) using ITLC after purification, and the results were shown in Fig.5. The radiochemical purity was more than 90% after incubating in eluant for 6h, although there was a slight falling down with the incubation time (Fig.6). The radiolabeling yield was only (10.68±3.15)% (n=6) when using $^{99m}TcO_4^-$ to label Annexin V directly without HYNIC conjugating. These results suggested the successful protein labeling with the bifunctional chelating agent, HYNIC.

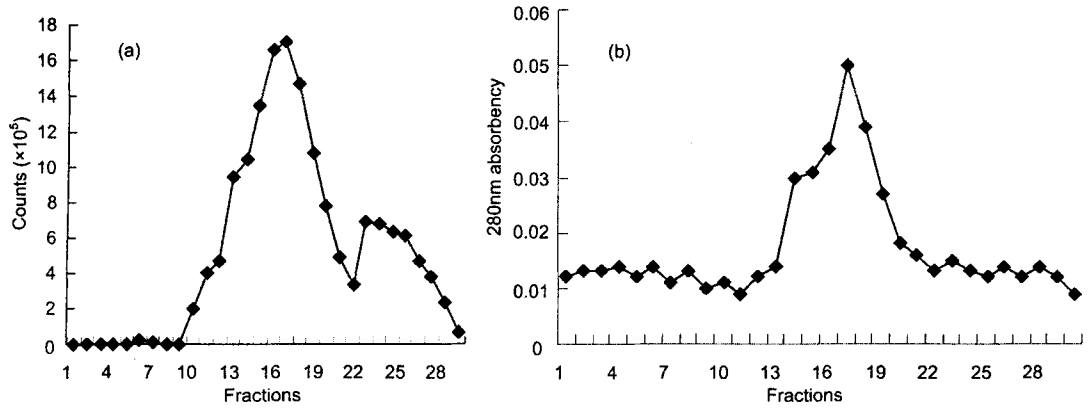

Fig.4 Results of (a) radioactivity counts and (b) the 280nm absorbency after purification by Sephadex G25.

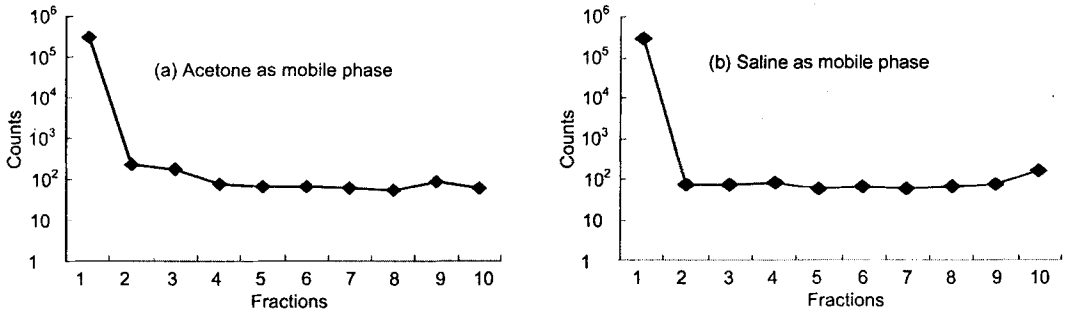

Fig.5 Results of radiochemical purity determination.

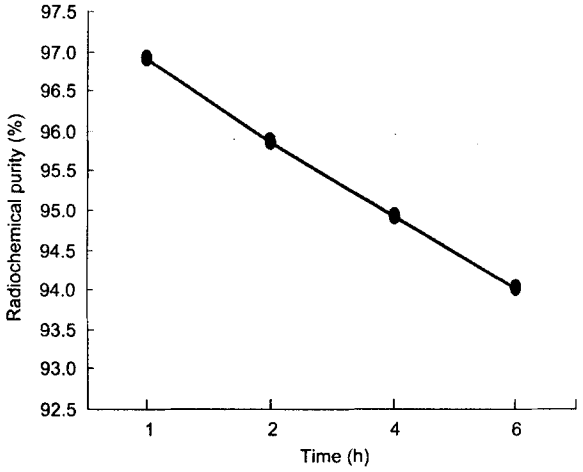

Fig.6 Stability of ^{99m}Tc-HYNIC-Annexin V.

4 Discussion

Programmed cell death plays an important role in many disease processes. Once the cell death sequence is initiated, cells undergoing apoptosis exhibit a stereotyped sequence of changing. Early in the course of apoptosis the lipid distribution in the plasma membrane is altered. A membrane-bound phospholipid, phosphatidylserine (PS), ordinarily confined to the inner leaflet of the plasma membrane by enzyme, translocase and scramblase, suddenly appears on the outer leaflet. Expression of PS on the cell membrane is quickly followed by DNA laddering and membrane bleb formation, both of which characterize apoptotic cell death.[4] Therefore, the expression of PS on the cell membrane is the early symbol of cell apoptosis, which makes PS as the target for early detecting apoptosis.

Annexin V is a human calcium-dependent endogenous protein with a molecular weight of 36kD. Annexin V has a high affinity (K_d=7nmol) for cell membranes with bound PS. Annexin V, therefore, is a sensitive marker of the early to intermediate phases of apoptosis. In vitro assays have been developed using fluorescence labeled Annexin V to detect apoptosis. Recently, radiolabeled Annexin V has been developed for detecting apoptosis in vivo,[7,8] which makes it possible to use apoptosis radionuclide imaging for diagnosis of disease and as instructions for clinical treatment.

Annexin V cannot be supplied in a large amount because of low natural source, which limits its usage in radionuclide imaging. In this study, we used PCR primer, designed by ourselves, to enlarge the coding sequence of Annexin V. The amount of bases of pro-

duction was the same as the expected one. The inserted gene sequence was the same as the published sequence, which suggested that the construction of Annexin V was successful. The results of SDS-PAGE and Western Blotting showed that Annexin V could be expressed stably in *E. coli*. In this study, large amount of protein was expressed and Ni-NTA Superflow was used to purify the protein. High purity and concentration of Annexin V could be gained, which was the important basis for radiolabeling.

In the studies of Annexin V radiolabeling, the most commonly used methods are indirect labeling using bifunctional chelating agents. Several chelating agents can be used in Annexin V radiolabeling, however, different conjugating agents can effect the characteristics of different labeling productions. In ^{99m}Tc-i-Annexin V, Annexin V was conjugated with n-1-imino-4-mercaptobutytyl side chain [9]. The radiochemical purity and stability were found to be suboptimal because the bifunctional ligand was only monodentate. In ^{99m}Tc-BTAP- Annexin V, the protein was modified with an N2S2 4,5-bis(thioacetamido) pentanoyl group. ^{99m}Tc-BTAP- Annexin V was stable and well defined but had to be prepared through a preformed chelate approach that was consuming.[10]

HYNIC, as an effective bifunctional chelating agent used from 1990s, had very good effect on radiolabeling DNA, RNA, peptide, antibody and so on.[11] In this study, HYNIC, synthesized by ourselves and identified by NMR, was used as a chelating agent. Annexin V is a relatively large molecule (Mr=36kD) and derivation with a limited number of HYNIC molecules, which had less influence on the overall structure and function of the protein.[12] The conditions for conjugating and labeling were moderate, the methods were simple. In the process of labeling, $SnCl_2$ was used as reducing agent and tricine as the co-ligand. The radiolabeling yield could reach 90% and radiochemical purity was more than 90%, which could meet the needs of imaging. But without conjugating, the radiolabeling yield was very low. Hence, HYNIC is one of the most attractive bifunctional agents for the labeling of protein with ^{99m}Tc.

5 Conclusion

In this study, Annexin V, a protein which can specifically bind with PS on apoptosis cell membrane, was prepared by genetic engineering with high yield and purity. Annexin V was successfully labeled with ^{99m}Tc using bifunctional chelating agent HYNIC, the radiolabeling yield and radiochemical purity satisfied the needs for radionuclide imaging. Preparation of ^{99m}Tc-HYNIC-Annexin V is the basis for apoptosis imaging in vivo, which has good clinical perspective.

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