



Cellular and Molecular Biology

Original Article

^{99m}Tc-HYNIC-Annexin V detection of apoptosis in the rabbit model of liver cancer

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Article Info

Abstract



Article history:

Received: February 14, 2024

Accepted: May 15, 2024

Published: July 31, 2024

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To investigate the feasibility of detection of apoptosis *in vivo* by ^{99m}Tc-HYNIC-Annexin V, Annexin V was labeled with ^{99m}Tc through HYNIC. 18 New Zealand rabbits implanted VX-2 were randomly divided into control (n = 8) and paclitaxel (PAC, n = 10) groups, given 2 mL/kg of normal saline or 2.4 mg/kg of PAC intravenously. The liver tumor imaging was detected by SPECT through intravenous injection of ^{99m}Tc-HYNIC-Annexin V before treatment, 24 hours and 48 hours after treatment respectively. Tumor radioactive count proportion to non-tumor sites was calculated. When the last imaging was finished, the rabbits were sacrificed. The tumor was taken out and divided into two pieces, one for TUNEL immunohistochemical analysis and the other for flow cytometry (FCM). We found that the rate of Annexin V labeled with ^{99m}Tc through HYNIC was more than 95%, and radiochemical purity was above 95%. The SPECT showed that two groups had no significant tumor imaging before the treatment. There is no significant tumor imaging in control group, while the PAC group 24 h and 48 h after treatment showed significant accumulation. The Tumor/non-Tumor (T/NT) in PAC group at 24 h and 48 h after chemotherapy was significantly different from that in the control group and PAC group prior to treatment. There was no significant difference between 24 h and 48 h in PAC group. The TUNEL-positive cells detected by immunohistochemistry and apoptotic rate detected by FCM in PAC group were significant different from those in control group. The T/NT was significantly correlated to TUNEL-positive cells and apoptotic rate of the tumor. PAC can induce apoptosis of rabbit VX-2 liver cancer cells. 24-48 h after paclitaxel chemotherapy is a window time for apoptosis detection. Apoptotic cells *in vivo* can be detected by SPECT through ^{99m}Tc-HYNIC-Annexin V.

Keywords: ^{99m}Tc-HYNIC-Annexin V, Cell apoptosis, Liver cancer, Paclitaxel, Single photon emission computed tomography, VX-2

1. Introduction

Apoptosis is the process of programmed cell death by various stimulations *in vitro* and *in vivo*. Apoptosis is very important for maintaining body balance and functional immune system and so on. Abnormal apoptosis has been shown to play an important role in a number of diseases such as tumors, autoimmune diseases, and viral infections [1]. The detection of apoptosis is mainly performed by DNA fragmentation analysis *in vitro*, apoptotic cell membrane change, apoptosis-related protein analysis, and apoptosis enzyme analysis [2, 3]. Regarding the fact that it is hard to get *in vivo* samples in many diseases, it will be very helpful for the diagnosis and prognosis if we can evaluate the apoptosis status *in vivo*.

Phosphatidylserine (PS) is an intrinsic phospholipid component of the lipid bilayer of the cell membrane, which is confined to the inner layer of the cell membrane by the combined action of translocase and floppase at normal physiological Ca⁺ concentrations [4]. In the early stage of apoptosis, scramblase is activated and the activity of translocase is inhibited, causing PS to migrate from the inner to the outer layer of the cell membrane and to be exposed

on the surface of the cell membrane [5]. While Annexin V, one of the superfamilies of calcium- as well as phospholipid-binding membrane-bound proteins, can be exposed to the apoptotic cell membrane surface of the PS tight binding, with radionuclide labelling Annexin V, can be detected *in vitro* its distribution *in vivo*, to achieve non-invasive detection of apoptosis purpose [6, 7]. Because the PS flap in the apoptotic process appears significantly earlier than the degradation of DNA and recognisable morphological changes in the cell, so in addition to non-invasive, early detection of apoptosis is also one of the advantages of this tracer. Radionuclide labelling Annexin V method has more reports, and the use of bifunctional chelator hydrazinonitotamide (HYNIC) method, 99m technetium (^{99m}Tc) labelling Annexin V, to obtain a high labelling rate and radiochemical purity of ^{99m}Tc-HYNIC-Annexin V, with better stability [8, 9]. And its biological distribution and radiation dose is ideal, no obvious toxic side effects in the human body, is a safe radiopharmaceutical [10]. Clinically, it enables the visualisation of apoptosis, which is of great significance for the treatment decision-making, efficacy monitoring and prognosis evaluation of tumour patients

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and the development of new anti-tumour drugs.

This study aimed to detect radioactive accumulation before, and after treatment with paclitaxel in rabbit liver cancer models using ^{99m}Tc-HYNIC-Annexin V, identify the apoptosis peak after chemotherapy, evaluate its relationship with TUNEL positive cell counts by immunohistochemistry and apoptosis rate analyzed by flow cytometry, and judge the value of ^{99m}Tc-HYNIC-Annexin V in apoptosis detection in rabbit liver cancer models.

2. Materials and methods

2.1. Animals and materials

18 male New Zealand rabbits (weighing 2.0-2.5 kg) were provided by the Animal Facility of Nanjing University. VX-2 cell line was offered by Prof. Xiaoping Chen (Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology). Dual probe SPECT was obtained from GE Millennium VG. Annexin V was provided by Nanjing University Life Science Institute. TUNEL kit was purchased from Boehringer Mannheim. Apoptosis detection Kit (FITC-Annexin V/PI) was purchased from Jingmei Biotech and ARP-labeled goat anti-rabbit IgG was purchased from Zhongshan Biotech Co. Paclitaxel was obtained from Sihuan Pharmaceutical Co.

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

HYNIC was synthesized following the procedure of Verbeke et al. [11]. HYNIC was coupled with Annexin V [12]. HYNIC-Annexin V was then labeled with ^{99m}Tc. After labeling, the labeling rate and radiochemical purity were detected by HPLC [13].

2.3. Establishment of rabbit VX-2 liver cancer models

18 New Zealand rabbits were anesthetized (promethazine 10mg/kg, ketamine 25mg/kg, i.m.) and then fixed on the operation board. When the rabbit liver was exposed, ophthalmology forceps were used to puncture substantial liver tissue, thus sinuses formed due to dilation. Two tumor pieces (come from the VX-2 rabbit), about 1mm in diameter, were inserted into the sinus. Gelatin sponge was used to close the sinus and stop bleeding. After two weeks, the formation of liver tumor was confirmed by Doppler ultrasound and CT. Tumor-bearing rabbits were randomly divided into 2 groups: control group (2ml/kg saline, i.v., n = 8) and paclitaxel group (2.4 mg/kg, i.v., n = 10).

2.4. ^{99m}Tc-HYNIC-Annexin V imaging

925-1110MBq (25-30mci) ^{99m}Tc-HYNIC-Annexin V was injected through the auricular vein 2 hours prior to paclitaxel treatment, 24 hours or 48 hours after chemotherapy respectively. Rabbits were anesthetized 2 hours later for imaging at supine position. Tumor radioactive (T) and leg non-tumor radioactive (NT) were counted. The T/NT ratio was calculated.

2.5. TUNEL-positive cells by immunohistochemistry

After imaging, the rabbits were sacrificed. The tumor was then taken out and divided into two pieces. One half was fixed with 10 % formalin for 24 h and paraffin-embedded for TUNEL immunohistochemical analysis and the other half for flow cytometry (FCM) analysis. TUNEL solution was prepared according to the manufacturer's instructions. The detailed procedure followed the manu-

facturer's instructions. The brown-positive staining cells were counted.

2.6. Apoptotic rate by flow cytometry

Single-cell suspensions from tumors were prepared by a mechanical enzymatic technique. The apoptotic rate was detected using Annexin V/PI Apoptosis detection kit.

2.7. Statistical analysis

Data were presented using mean ± standard deviation. Comparison between 2 groups was evaluated by t-test. Multiple-group comparison was evaluated with ANOVA. The statistical significance of correlation between different factors was evaluated by Pearson correlation analysis. A P value of less than 0.05 was considered to be statistically significant. All the data were analyzed using SPSS17.0.

3. Results

3.1. Rabbit VX-2 liver cancer models

The labeling rate of ^{99m}Tc-HYNIC-Annexin V was more than 95%, and its radiochemical purity was above 95%. The labeling rate remained more than 85% after 6 h in vitro. The liver tumor can be observed 2 weeks after implantation. The tumor margin is clear and the blood supply is sufficient. The tumor diameter is 2-3 cm (Fig. 1). The tumor uptake rate is 100%.

3.2. ^{99m}Tc-HYNIC-Annexin V labeling in the different treatment groups, correlation of ^{99m}Tc-HYNIC-Annexin V labeling and TUNEL, FCM

There was no significant tumor imaging in control group, while the PAC group at 24 h and 48 h after treatment showed significant accumulation (Fig. 2). The Tumor/non-Tumor (T/NT) of PAC group after treatment for 24 h and 48 h were significantly different from that of the control group and PAC group prior to treatment (Table 1, P < 0.01). There was no significant difference between 24 h and 48 h after treatment in PAC group (P > 0.05).

The TUNEL-positive cells detected by immunohistochemistry and apoptotic rate detected by FCM in PAC group (Fig. 3A-E) were significant different from that in control group (P < 0.01, Table 2). The T/NT was signifi-

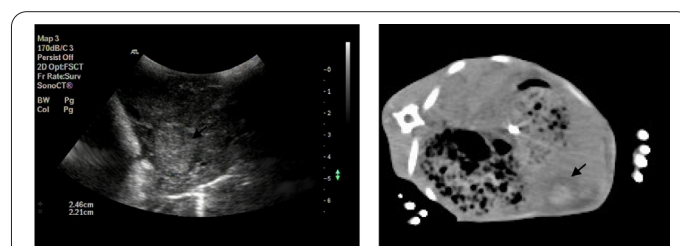


Fig. 1. Representative Doppler ultrasound and CT images of rabbit VX-2 liver.



Fig. 2. Representative SPECT imaging of different treatment groups.

Table 1. Comparison of the T/NT after chemotherapy for 0, 24 or 48 h.

Time/drug	Control (n=8)	PAC (n=10)
0 h	2.62±0.56	2.53±0.60
24 h	2.72±0.70	6.43±0.97 ^{a, b}
48 h	2.62±0.66	6.64±1.15 ^{a, b}

Notes: a. significant difference compared with control group; b. significant difference compared with before paclitaxel treatment, *P* < 0.01.

Table 2. Result of Immunohistochemistry and FCM.

Group	Control (n=8)	PAC (n=10)
Apoptotic cells/mm ²	4±0.81	13.50±2.74
Apoptotic rate %	7.83±1.11	20.81±3.33

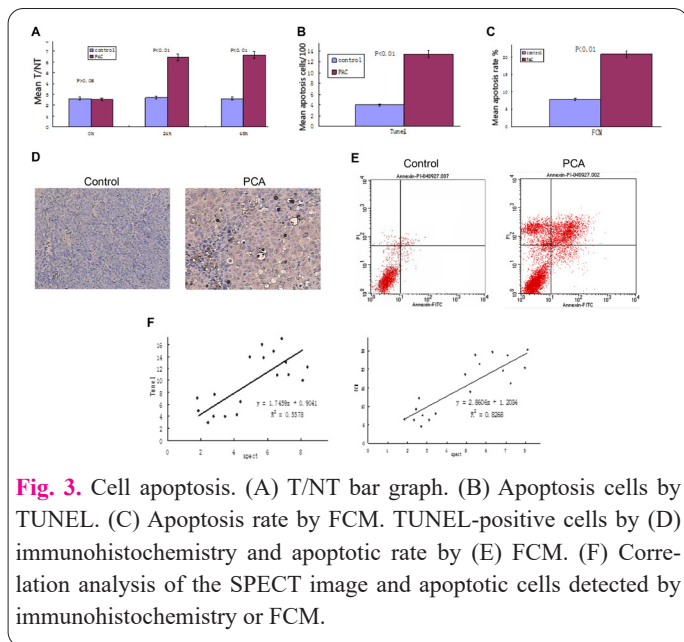


Fig. 3. Cell apoptosis. (A) T/NT bar graph. (B) Apoptosis cells by TUNEL. (C) Apoptosis rate by FCM. TUNEL-positive cells by (D) immunohistochemistry and apoptotic rate by (E) FCM. (F) Correlation analysis of the SPECT image and apoptotic cells detected by immunohistochemistry or FCM.

cantly correlated to TUNEL-positive cells and apoptotic rate of the tumor (Fig. 3F) $R_{TUNEL} = 0.747, P = 0.013$; $R_{FCM} = 0.909, P < 0.01$.

4. Discussion

PS is a kind of phospholipid with negative charge, usually kept on the cytosolic side of cell membranes by an enzyme called flippase. When a cell undergoes apoptosis, PS is no longer restricted to the cytosolic part of the membrane but becomes exposed on the cell surface [14]. Annexin V has high affinity with PS on the apoptotic cells' surface [15]. When it is marked with a fluorescent or enzymatic label, biotin or other tags, it can be used to detect apoptotic cells through flow cytometry analysis or fluorescence microscope. HYNIC has relatively small molecular weight compared with Annexin V and won't affect the total molecular weight and structure of the coupling protein [11]. HYNIC-Annexin V can easily be labeled with ^{99m}Tc by simple addition of Sn²⁺ ions and pertechnetate in the presence of tricaine as coligand and results in preparations with radiochemical yields exceeding 90% in only 15 minutes [10]. Kuge et al. reported that there was significant difference between the pre-treatment group and post-treatment group in a rat model through ^{99m}Tc-HYNIC-Annexin V labeling [16]. Hoebbers, Bellhocine, and Blankenberg found that, through ^{99m}Tc-HYNIC-Annexin V SPECT in patients, there were significant accumulation counts in the target organ after treatment [17-19].

Paclitaxel is a common cytoskeletal anti-cancer drug used in the clinic. In our study, ^{99m}Tc-HYNIC-Annexin V was injected into the rabbit through the auricular vein 2 hours prior to paclitaxel treatment, 24 hours and 48 hours after chemotherapy respectively. The SPECT showed that there was no significant tumor imaging in control group, while the PAC group which was performed 24 hours and 48 hours after treatment showed significant accumulation. The Tumor/non-Tumor (T/NT) of PAC group (24 hours and 48 hours after treatment respectively) was significantly distinguished from that of the control group and also PAC group (prior to treatment). In the PAC group, 24 hours and 48 hours after the treatment, no significant difference was detected in the SPECT imaging. The TUNEL-positive cells detected by immunohistochemistry and apoptotic rate detected by FCM in PAC group were significant different from that in control group. The T/NT was significantly correlated to TUNEL-positive cells and apoptotic rate of the tumor. Our data demonstrated that PAC can induce apoptosis of rabbit VX-2 liver cancer cells. Apoptotic cells in vivo can be detected by SPECT through ^{99m}Tc-HYNIC-Annexin V, which was consistent with the results of immunohistochemistry and FCM.

Vermeersch et al. have pointed out that tumor tissue has abundant blood supply, and the local blood supply might affect accumulation of radionuclides [20]. But the blood supply of liver tumor has the feature of quick circulation. ^{99m}Tc-HYNIC-Annexin V also has a relatively long half-life period and SPECT imaging was performed 2 hours after the injection of paclitaxel, thus minimizing the effects of the local blood supply. ^{99m}Tc-HYNIC-Annexin V is excreted mainly through the kidneys, rarely through the liver or digestive tract. Our study has shown that before the injection of paclitaxel, ^{99m}Tc-HYNIC-Annexin V accumulated in both kidneys and bladder obviously, while in other organs especially the digestive tract, there was few. The results were consistent with the study by Kemerink et al. [10]. The same method may be used in other cancers in the future [21-26]. The drug metabolism did not affect the imaging of the liver tumor, so ^{99m}Tc-HYNIC-Annexin V can be used for detecting apoptosis after the treatment of the liver tumor.

The tumor cell apoptosis peak is usually 24 -72 hours after chemotherapy. Our study showed that there was no significant difference between the post-therapy 24-hour point and 48 hours point. It suggested that the radioactive uptake may reach a plateau, which correlated to the peak of chemotherapy effect.

5. Conclusion

We concluded that PAC can induce the apoptosis of rabbit VX-2 liver cancer cells. 24-48 hours after paclitaxel chemotherapy is a window time for apoptosis detection. SPECT imaging through ^{99m}Tc-HYNIC-Annexin V can detect apoptosis in vivo. This method can evaluate the apoptotic effects induced by chemotherapy drugs and provides a new avenue for chemotherapeutics selection in the clinic.

Conflict of Interests

The authors declare no competing interests.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

We have received approval from the Ethics Committee of Jiangning Hospital Affiliated to Nanjing Medical University.

Informed Consent

Not applicable.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

HM contributed to the study conception and design. Experimental operation, data collection and analysis were performed by SZ and BJ. The first draft of the manuscript was written by SZ and all authors commented on previous versions of the manuscript.

Funding

The current study was supported by Key Topic of the Nanjing Medical Science and Technology Development Project (No. ZKXO7004).

Acknowledgement

Not applicable.

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