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Study of ^{99m}Tc-annexin V uptake in apoptotic cell models of Parkinson's disease

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Objective **To investigate the feasibility of radiolabelled annexin V imaging for early diagnosis of Parkinson's disease.**

Methods **^{99m}Tc-HYNIC-annexin V was prepared and its binding to apoptotic cell models of Parkinson's disease was studied *in vitro*. Cellular models of Parkinson's disease were produced by administering different concentrations of 1-methyl-4-phenylpyridinium to PC12 and SH-SY5Y cell lines. Cell apoptosis rates were analysed by flow cytometry. Annexin V was labelled with ^{99m}Tc by hydrazinonicotinamide (HYNIC). Cell binding studies were carried out using cellular models of Parkinson's disease. Cell uptake studies were also performed after different levels of MPP^+ treatment, and the correlation between the degree of apoptosis and ^{99m}Tc-HYNIC-annexin V uptake was analysed.**

Results **The specific activity of ^{99m}Tc-HYNIC-annexin V was $3.7–74 \times 10^5$ Bq·mg^{-1} protein. In-vitro binding of ^{99m}Tc-HYNIC-annexin V to model cells was specific, saturable and time dependent. Scatchard analysis gave a K_d of 7.16 ± 1.78 nmol·l^{-1}, B_{max} values of 179 ± 33 fmol per 10^6 cells (PC12) and 220 ± 26 fmol per 10^6 cells (SHSY5Y). MPP^+ at different concentrations can induce cell apoptosis in a dose-dependent manner; cellular uptake of ^{99m}Tc-HYNIC-annexin V as indicated by membrane-bound radiolabelled annexin V activity was linearly correlated with total fluorescence, as observed by FITC-annexin V flow cytometry (PC12: $r=0.924$; SH-SY5Y: $r=0.937$, $P<0.01$).**

Conclusions **^{99m}Tc-HYNIC-annexin V retains its receptor-binding activity and has a high affinity to cellular models of Parkinson's disease. The uptake of radioactivity correlated well with cell apoptosis rates; thus, ^{99m}Tc-annexin V is a potential imaging agent with which to detect early neuron damage in Parkinson's disease. *Nucl Med Commun* 28:895–901 © 2007 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Keywords: Parkinson's disease, annexin V, apoptosis imaging radioligand binding assay

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Introduction

Parkinson's disease (PD), the second most common human neurodegenerative disorder after Alzheimer's disease, is characterized by tremors at rest, bradykinesia, rigidity, postural instability and an expressionless face.

Although the pathogenesis of PD remains unclear, there is evidence that apoptosis may play an important role in the selective loss of dopaminergic neurons in the substantia nigra pars compacta [1]. Annexin V, an endogenous protein, can bind selectively to phosphatidylserine on the surfaces of apoptotic cells. Fluorescently labelled and biotinylated annexin V is now widely used for the in-vitro detection of cell apoptosis using immunohistochemistry, microscopy and flow cytometry [2]. However, all of these detection methods are restricted to in-vitro applications, and usually require biopsies or invasive sampling. Recently, annexin V has been radiolabelled with radionuclides such as ^{125}I, ^{18}F and ^{99m}Tc, for the non-invasive detection of apoptosis *in vivo* [3–5]. It is a promising radiopharmaceutical agent for in-vivo imaging of apoptosis and is now under clinical trials for non-invasive evaluation of tumour apoptosis after chemotherapeutics and monitoring of chemosensitivity [6,7]. D'Arceuil [8] indicated that radiolabelled annexin V can bind to apoptotic neurons in individuals with an intact blood–brain barrier (BBB), enabling the early detection of apoptotic dopaminergic neurons in PD patients. Meanwhile, if apoptosis is detected early enough, the early administration of a neuroprotective agent may block the apoptotic cascade, because apoptosis is a multistep process that lasts for a significant amount of time.

In this study, we tried to determine whether radiolabelled annexin V could be used to non-invasively monitor neuronal apoptosis in cellular models of Parkinson's

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disease, and assess the utility of ^{99m}Tc-annexin V imaging for the early diagnosis of the disease.

Materials and methods

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

Human recombinant annexin V was produced by expression in *Escherichia coli* as described previously [9,10]. Hydrazinonicotinamido (HYNIC), a bifunctional linker molecule with one moiety that binds to a protein lysine residue and another that binds to complexes of ^{99m}Tc was obtained from 6-chloronicotinic acid according to the methods of Abrams *et al.* [11] and Blankenberg *et al.* [12], via four steps: hydrazine replacement, *tert*-butoxycarbonylation (BOC), *N*-hydroxysuccinimidyl connection and BOC removal. To couple HYNIC to annexin V, 42 mmol·l^{-1} HYNIC in *N*,*N*-dimethylformamide was mixed with 3 mg·ml^{-1} annexin V in phosphate buffered saline (PBS, pH 7.4) for 3 h, shielded from light, at room temperature. The reaction was quenched by 500 mmol·l^{-1} glycine in PBS. Then HYNIC-conjugated annexin V was dialysed overnight at 4°C against 20 mmol·l^{-1} sodium citrate, and 100 mmol·l^{-1} NaCl, pH 5.2. The precipitate was removed by centrifugation at 15 000 × *g* for 10 min. Aliquots (100 µl) of HYNIC-annexin V were stored at 20°C before use.

Radiolabelling with ^{99m}Tc was performed by adding [^{99m}Tc]pertechnetate solution (37–370 MBq), Sn-tricine solution (200 µl of 100 mg·ml^{-1} tricine in 20 mmol·l^{-1} citrate buffer, pH 5.2, and 1 mg·ml^{-1} $SnCl_2 \cdot 2H_2O$ in 0.05 mol·l^{-1} HCl) to HYNIC-conjugated annexin V. Sn-tricine was prepared just before use. The mixture was incubated at room temperature for 15 min and then purified by a 0.7 cm × 20 cm Sephadex gel filtration column (G-25, Pharmacia) using 0.01 mol·l^{-1} PBS (pH 7.4) with 2 mg·ml^{-1} tricine as eluant. The radiochemical purity was determined by instant thin-layer chromatography (ITLC) using 0.9% saline solution as a solvent. ^{99m}Tc-HYNIC-annexin V was incubated in the eluant for 1, 2, 4 or 6 h, and ITLC was performed to evaluate the stability. For further study, ^{99m}Tc-HYNIC-annexin V was diluted with saline to give the appropriate protein concentration or amount of radioactivity.

Cell culture

The rat pheochromocytoma cell line PC12 and the human neuroblastoma cell line SH-SY5Y were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO Invitrogen Corporation) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U·ml^{-1} penicillin and 100 µg·ml^{-1} streptomycin. Cells were maintained as monolayers in a humidified 5% carbon dioxide atmosphere at 37°C. The PC12 cells were differentiated into neurons with 100 ng·ml^{-1} nerve growth factor in DMEM. The cells were separated for subculture using 0.25% trypsin (Amresco), resuspended in fresh medium containing fetal calf serum, and replanted in 6-well or 24-well culture plates for further study.

Cellular models of Parkinson's disease

1-methyl-4-phenylpyridinium (MPP^+) (Sigma), an active neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), can mimic neuron loss in the nigrostriatal dopaminergic system. It is still the best characterized toxin paradigm for Parkinson's disease, faithfully replicating most of PD's clinical and pathological hallmarks. The toxicity of MPP^+ is mediated through ATP depletion, mitochondrial complex I inhibition, upregulation of oxygen radicals and loss of mitochondrial membrane potential [13,14]. There is evidence that the cell death induced by MPP^+ is apoptotic. To reproduce apoptotic cell models of Parkinson's disease, PC12 and SH-SY5Y cells at 70–80% confluence were treated with MPP^+ at different concentrations (PC12 cells, 0–200 µmol·l^{-1}; SH-SY5Y cells, 0–500 µmol·l^{-1}). After incubation for 48 h (PC12 cells) or 72 h (SH-SY5Y cells), cells were collected and media were refreshed for further study. MPP^+ solutions were prepared shielded from light in DMEM just before use.

Flow cytometry analysis

The rates of cell apoptosis were analysed by flow cytometry (Becton Dickinson) following fluorescein isothiocyanate-conjugated annexin V (FITC-annexin V) and propidium iodide (PI) staining. FITC-annexin V was used to detect exposed phosphatidylserine moieties on the membrane during the early stages of apoptosis; PI is a fluorescent biomolecule that can permeate damaged membranes and stain DNA, but which is excluded from viable cells. It is commonly used to identify necrotic cells or cells in the late stages of apoptosis. The staining procedure was performed according to the instructions of the annexin V-FITC Apoptosis Detection Kit (BioVision). After the indicated time periods following MPP^+ induction, cells were gently trypsinized and washed once with serum-containing media. Then, 1–5 × 10^5 cells were collected and washed twice with cold PBS by gentle shaking. Cells were resuspended in 500 µl of prediluted 1 × binding buffer and then 10 µl of annexin V-FITC and 5 µl of propidium iodide were added. The cell suspension was gently vortexed and incubated at room temperature for 15 min, shielded from light. Flow cytometry was performed as soon as possible (within 1 h). In the annexin V–PI co-labelling method, the annexin V-negative/PI-negative fraction represented viable cells, the annexin V-positive/PI-negative fraction represented early apoptotic cells, and the annexin V-positive/PI-positive fraction represented late apoptotic and dead cells.

Binding studies

A cell binding assay was performed according to the method of Stern *et al.*, with some minor modifications

[15–17]. In brief, to assess time and temperature influences, MPP^+-stimulated cells in 24-well plastic plates were washed with HEPES buffer (150 mmol·l^{-1} NaCl, 4 mmol·l^{-1} KCl, 10 mmol·l^{-1} HEPES, 11 mmol·l^{-1} glucose, pH 7.45) containing 1 mmol·l^{-1} EDTA to remove possible traces of endogenous annexin V present in the culture medium. The cells were resuspended in HEPES buffer containing 5 mmol·l^{-1} Ca^{2+} and 1 mg·ml^{-1} bovine serum albumin (BSA, Amresco). Subsequently, duplicate wells were incubated with ^{99m}Tc-HYNIC-annexin V at different temperatures (4°C, 20°C, 37°C) for different time periods, with constant oscillation on an orbit shaker (60 oscillations per min). At harvesting, cell samples were collected and washed twice with ice-cold HEPES buffer containing 5 mmol·l^{-1} $CaCl_2$. Unbound ^{99m}Tc-HYNIC-annexin V was removed by centrifugation and the amount of cell-associated radioligand was determined using a gamma counter. For binding assays, cells were collected and incubated with a wide range of concentrations of ^{99m}Tc-HYNIC-annexin V (0–50 nmol·l^{-1}). Specific binding was determined by subtracting the non-specific binding, which was determined in the presence of a 1000-fold excess of unlabelled annexin V. The dissociation constants and the number of binding sites were analysed by Scatchard's method. Apparent dissociation constants and the number of binding sites were calculated in Prism 4. For displacement competition test, cells were incubated with ^{99m}Tc-HYNIC-annexin V and different concentrations of unlabelled annexin V. The cellular uptake of ^{99m}Tc-HYNIC-annexin V was also analysed. Data are the averages of duplicate determinations.

In-vitro cellular uptake assay

To quantify ^{99m}Tc-HYNIC-annexin V uptake in cells undergoing different degrees of apoptosis during MPP^+ incubation, a cellular uptake test was carried out as previously described [18].^{99m}Tc-HYNIC-annexin V (37–74 kBq per well) was added to 24-well flat-bottomed plates at 1 ml per well, and incubated at 37°C for 90 min. Cells were harvested and cell-associated activities were measured in a gamma counter. Simultaneously, the same amount of cell was harvested and stained using FITC-annexin V and PI for flow cytometry quantification. The correlation between the degree of apoptosis (as detected by flow cytometry) and cellular uptake of ^{99m}Tc-HYNIC-annexin V was analysed. Data represent the averages of four measurements.

Results

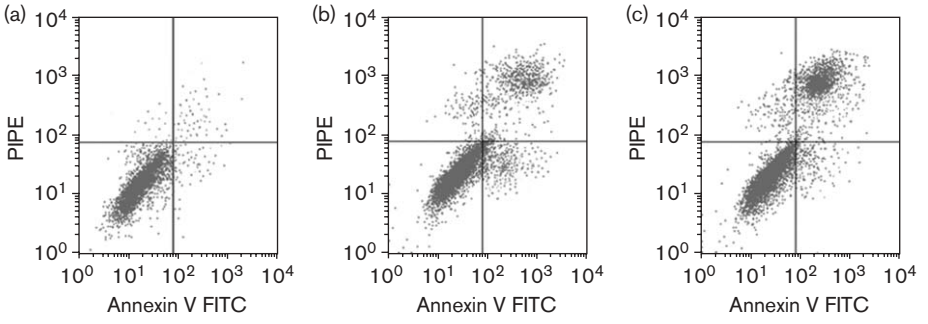
Coupling and radiolabelling

The specific activity of ^{99m}Tc-HYNIC-annexin V was 3.7–74 $\times 10^5$ Bq·mg^{-1} protein. Radiochemical purity was above 95% after purification. The stability test of ^{99m}Tc-HYNIC-annexin V indicated that the radiochemical purity remained above 90% after 6 h storage at room temperature.

Flow cytometry analysis

Following annexin V and PI double staining, flow cytometry identified four distinct populations (see Fig. 1c for an example): double-negative cells (AV^-/PI^-), located in the lower left quadrants, represent viable cells; FITC-annexin V-positive, PI-negative cells (AV^+/PI^-), located in the lower right quadrant, represent early stage apoptotic cells; double-positive cells (AV^+/PI^+), located in the upper right quadrant, represent late stage apoptotic cells and necrotic cells; and PI-positive, AV-negative cells (AV^-/PI^+), located in the upper left quadrant, represent cells that were damaged during the procedure. Flow cytometry measurements show that MPP^+ induces cell apoptosis in a dose-dependent manner, as shown in Table 1 and Figs 1 and 2. With increasing MPP^+ concentration, the ratio of late-stage apoptotic cells and necrotic cells (upper right quadrant) increased. For early detection of cell apoptosis, binding

Fig. 1



Flow cytometric analysis of apoptotic cells using FITC-annexin V and PI stain. (a) Untreated cells were primarily FITC-annexin V and PI negative (lower left quadrant), indicating that the cells were viable and not undergoing apoptosis. (b) After lower concentration treatment of MPP^+ (20 μmol·l^{-1}), a significant number of cells are FITC-annexin V positive and PI negative (lower right quadrant), indicating that the cells are in an early stage of apoptosis and are still viable. (c) After higher concentration treatment of MPP^+ (100 μmol·l^{-1}), a population of cells has progressed to a later stage of apoptosis and is stained with both PI and annexin V (upper right quadrant), indicating that the cells are no longer viable.

studies were all performed under lower MPP^+ concentration (20 μm for PC12 cells and 50 μm for SH-SY5Y cells). At even lower concentrations, few cells were induced to undergo apoptosis by MPP^+.

Time course of ^{99m}Tc-HYNIC-annexin V binding

Binding of ^{99m}Tc-HYNIC-annexin V to PC12 and SH-SY5Y cells reached an apparent maximum after 60–90 min of incubation at 37°C. The initial rate of annexin V binding was slower at 4°C than at 20°C and 37°C; at 60 min, nearly the same amount of ^{99m}Tc-HYNIC-annexin V was bound to the cell models. Since the rate and extent of annexin V binding to the cell surface were similar at different temperatures, all further experiments were carried out at 37°C.

Table 1 Apoptotic cell fraction after different concentrations of MPP^+ treatment ($n=3$, X ± s %)

MPP^+ concentration ($\mu mol \cdot l^{-1}$)	Apoptosis fraction (%)	
	PC-12	SH-SY5Y
Control	4.0 ± 1.6	1.2 ± 0.1
20	12.0 ± 1.4*	–
50	16.7 ± 1.4*	10.6 ± 1.2*
100	26.0 ± 2.6*	19.4 ± 1.3*
200	49.1 ± 6.9*	25.4 ± 2.3*
500	–	34.4 ± 2.5*

*$P<0.01$ vs. control.

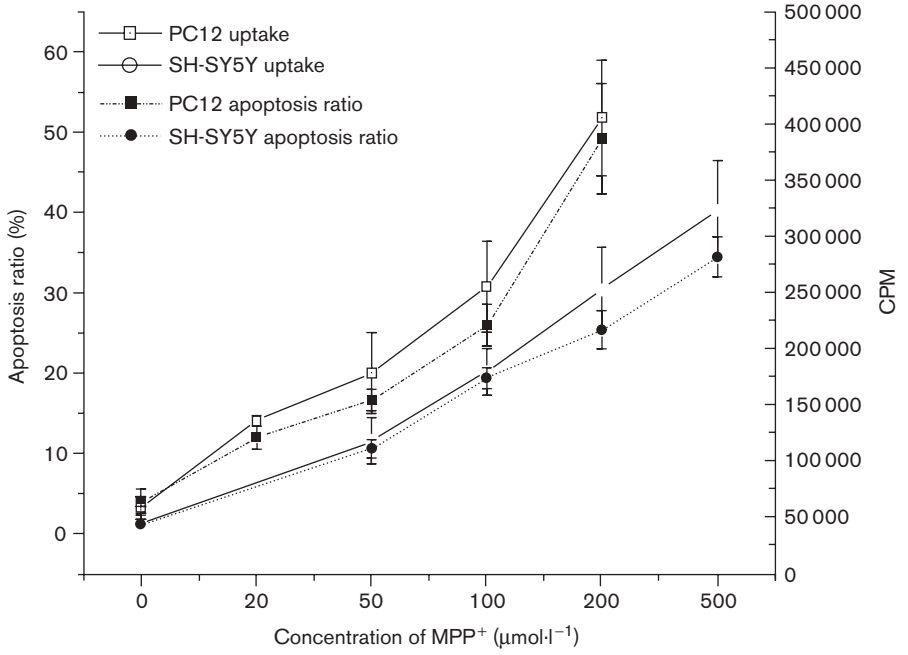
Saturability of binding

Dissociation constants (K_d) and the number of binding sites of annexin V to MPP^+-stimulated PC12 and SH-SY5Y cells were measured at various concentrations of labelled annexin V. Specific binding was determined by subtracting the amount of ^{99m}Tc-HYNIC-annexin V bound in the presence of a 1000-fold excess of unlabelled annexin V from the total amount of radioligand bound. Bound radioactivity was expressed as an average value of radioactivity observed in duplicate wells. The binding isotherm of ^{99m}Tc-HYNIC-annexin V to cells exhibited a sigmoidal profile, exhibiting concentration dependence, and approached saturation at approximately 40 $nmol \cdot l^{-1}$. Scatchard analysis of the data gives an apparent K_d of 7.16 ± 1.78 $nmol \cdot l^{-1}$ (mean ± SE, six experiments), B_{max} values of 179 ± 33 fmol per 10^6 cells (PC12 cells) and 220 ± 26 fmol per 10^6 cells (SH-SY5Y cells), specific binding sites of $8.96 \pm 0.16 \times 10^5$ binding sites/apoptotic PC12 cell and $1.25 \pm 0.14 \times 10^6$ binding sites/apoptotic SH-SY5Y cells.

Competition test

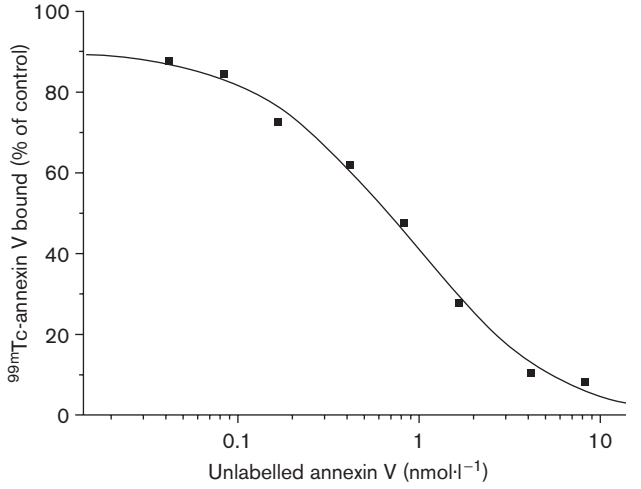
In the competition test, ^{99m}Tc-HYNIC-annexin V was incubated for 1 h at room temperature with cell monolayers

Fig. 2



PC12 and SH-SY5Y cells apoptosis ratio and ^{99m}Tc-HYNIC-annexin V uptake after MPP^+ treatment. PC12 and SH-SY5Y cells underwent different concentrations of MPP^+ treatment (PC12: 0, 20, 50, 100, 200 $\mu mol \cdot l^{-1}$; SH-SY5Y: 0, 50, 100, 200, 500 $\mu mol \cdot l^{-1}$). The percentages of cell apoptosis (FITC-annexin V-positive cells) and cell uptake of ^{99m}Tc-HYNIC-annexin V was compared. The bound radioactivity to duplicate cell samples are expressed as counts per minute (CPM). There was a markedly increased uptake of ^{99m}Tc-HYNIC-annexin V in MPP^+ treated groups compared to non-treated groups. (Relativity: PC12: $r=0.924$, $P<0.01$; SH-SY5Y: $r=0.937$, $P<0.01$; apoptosis ratio significance of difference PC-12 cells; 20, 50, 100, 200 $\mu mol \cdot l^{-1}$ vs. 0, $P<0.01$. SH-SY5Y cells: 20, 50, 100, 200 $\mu mol \cdot l^{-1}$ vs. 0, $P<0.01$).

Fig. 3



Competition of ^{99m}Tc-HYNIC-annexin V binding to PD cellular models by unlabelled annexin V. The percentage of ^{99m}Tc-annexin V bound activity was calculated from the observed bound activity divided by the bound activity in the absence of competitor.

in the presence of different amounts of unlabelled annexin V. The amount of non-specific binding was determined using a 1000-fold excess of unlabelled annexin V. The competition curve is shown in Fig. 3, indicating that the binding of ^{99m}Tc-HYNIC-annexin V to PC12 and SH-SY5Y cells was inhibited by unlabelled annexin V.

In-vitro cellular uptake assay

Treated and control cells were incubated at 37°C with 37 kBq of ^{99m}Tc-HYNIC-annexin V. Sixty minutes later, cells was harvested for uptake assays and flow cytometry analysis. Flow cytometry results obtained with FITC-annexin V and PI stain were compared with the radioactive uptake of ^{99m}Tc-HYNIC-annexin V in control and MPP^+-treated cells. The experiments were performed in triplicate. The cell uptake tests suggested that the membrane-bound radiolabelled annexin V activity was linearly correlated with total fluorescence, as observed by FITC-annexin V flow cytometry ($r = 0.924$ for PC12 cells, $r = 0.937$ for SH-SY5Y cells, $P < 0.01$).

Discussion

Parkinson's disease is prevalent all over the world in all ethnic groups, mainly affecting people older than 65 years of both sexes [19]. The diagnosis of PD is made mainly on the basis of strict clinical criteria. But misdiagnosis of PD is common. Research indicates that only 75% of clinical diagnoses of PD are confirmed at autopsy [20–22]. The reason for this lies in the fact that, on one hand, the symptoms of PD are not specific and may occur in many diseases like depression, essential tremor, Huntington disease, Wilson's disease, multiple system atrophy and progressive supranuclear palsy, as well as in drug users; and on the other hand, no special laboratory text and examinations are available for the diagnosis of PD, other than the exclusion of other conditions. The 'gold standard' for PD diagnosis remains neuropathological examination, which makes early and accurate diagnosis difficult before death. Meanwhile, more than 50% of nigral neurons and 80% of striatal dopamine are lost by the time the typical clinical signs and symptoms appear, which makes subsequent treatment quite difficult. Therefore, it is urgent to establish an effective diagnosis method for the early diagnosis of PD.

Although the subject of intensive research, the aetiology of PD remains enigmatic. Mitochondrial dysfunction, oxidative stress, the actions of excitotoxins, neurotrophic and immune factors are all suggested to be causes of PD. Regardless of the cause, the end result of PD is neuronal loss in the pars compacta of the substantia nigra. Recently, increasing evidence suggests that neuronal death in the pars compacta of the substantia nigra may be apoptotic [1,23]. Mochizuki *et al.* [24] studied the midbrains of some PD patients using the nick-end labelling method. Intense nuclear staining was observed in most PD patients, indicating that apoptotic processes may be a contributor to the nigral neuron death in PD. The molecular and biochemical pathways of apoptosis have also been found in related cellular and animal models of PD. Decreased concentrations of annexin V in parkinsonian cerebrospinal fluid also support the occurrence of neuronal apoptosis in PD [25].

Apoptosis is a multistep process, characterized by phosphatidylserine eversion, membrane blebbing, cell condensation, fragmentation of nuclear chromatin and formation of apoptotic bodies [26]. Earlier reports have shown that the phosphatidylserine, a membrane phospholipid normally restricted to the cytoplasmic side of the lipid bilayer, will flip-flop out during the early stages of apoptosis. The externalization of phosphatidylserine is the earliest event in apoptosis, while the internucleosomal DNA cleavage and plasma membrane permeabilization occur at the late stages. It is a general feature of cell apoptosis.

Annexin V, an endogenous human protein, is a sensitive probe for the study of phosphatidylserine externalization before the other well-described morphological or nuclear changes of apoptosis become detectable. Radiolabelled annexin V is under intensive study for use in the detection of apoptosis in myocardial ischaemia–reperfusion injury monitoring and prediction of anti-cancer treatment response [6,27–29]. It is the most promising radiopharmaceutical agent for in-vivo imaging of early events in apoptosis, and is now under clinical trials for the non-invasive evaluation of tumour apoptosis. In addition, D'Arceuil *et al.* [8] has shown that radiolabelled annexin V

can detect neuronal cell death with an intact blood–brain barrier, and this was confirmed by ^{111}In-diethylenetriaminepentaacetic acid (DTPA) radionuclide and Gd-DTPA magnetic resonance imaging. Mari *et al.* [30] used the same rodent model and confirmed that radiolabelled annexin V can be used for the detection of focal hypoxic–ischaemic injury in brain. Interestingly, they found that the radiolabelled annexin V can not only be used for detecting cell death, but can also be used for imaging neurons under stress, which may be a pre-apoptotic condition, or 'reversible' damage to neurons, causing transient expression of phosphatidylserine that may or may not be a signal of cell apoptosis later on. This information may enable the early detection of apoptosis in dopaminergic neurons or stressed neurons in PD patients. If apoptosis is detected earlier, neuroprotective agent administration and other treatments may block the apoptotic cascade and save neurons.

In light of this information, we tried to apply radiolabelled annexin V to the early diagnosis and evaluation of PD. In this study, the hydrazino-nicotinamide (HYNIC) ligand, a bifunctional chelating agent, was prepared for the conjunction of ^{99m}Tc and annexin V. The product was purified by Sephadex G-25 column chromatography and analysed by ITLC. The radiochemical purity was $>90\%$ after 6 h of storage at room temperature. The preparation of ^{99m}Tc-HYNIC-annexin V is easy and rapid; no additional purification step is needed. It is the only in-vivo apoptosis-detecting radioligand under phase II/III clinic trials in patients suffering from non-small-cell lung cancer, making ^{99m}Tc-HYNIC-annexin V one of the most promising apoptosis-detecting radioligands for routine production and clinical use [31,32].

MPP^+, an intermediate metabolite of MPTP, is widely used for the development of PD cell models. It is regarded as the best chemical for faithful replication of most of the clinical and pathological hallmarks of PD. In our study, MPP^+ caused PC12 and SH-SY5Y cell death in a dose-dependent and time-dependent manner. We did not observe much cell death when cells were treated with MPP^+ at different concentrations (0–200 $\mu mol \cdot l^{-1}$) for less than 48 h. However, incubations lasting longer than 48 h, with increasing of MPP^+ concentrations, resulted in a significant increase in the percentage of cell death. Meanwhile, the percentage of later stage apoptosis and necrosis also increased. In order to detect cells undergoing the early stages of cell death, a binding study was carried out in the presence of low concentrations of MPP^+ (20 $\mu mol \cdot l^{-1}$ 48 h for PC12 cells and 50 $\mu mol \cdot l^{-1}$ 72 h for SH-SY5Y cells). The in-vitro binding study suggested that ^{99m}Tc-HYNIC-annexin V retains its receptor-binding activity after radiolabelling, and the binding of ^{99m}Tc-HYNIC-annexin V to cellular models of Parkinson's disease was specific, saturable and time-dependent. No significant temperature dependence was observed. The association of annexin V with the cell surface was specific; excess quantities of unlabelled annexin V competed for radioligand binding. The apparent K_d of about 7 $nmol \cdot l^{-1}$ for annexin V binding to PD cellular models is in good agreement with earlier reports of determined K_d values for annexin V binding to phosphatidylserine-containing vesicles, activated platelets and OC-2008 cells. The numbers of annexin V binding sites on the PD cellular models were estimated to be $8.96 \pm 0.16 \times 10^5$ binding sites/apoptotic PC12 cell and $1.25 \pm 0.14 \times 10^6$ binding sites/apoptotic SH-SY5Y cell, which is different from the number of sites reported for activated human platelets and OC-2008 cells. This presumably reflects the difference in cell types and agonists [33,34]. In our study, membrane-bound radiolabelled annexin V activity and FITC-annexin V flow cytometry measurements were compared. The data demonstrated that there is a statistically significant correlation between the apoptosis rates measured by the two techniques for detecting MPP^+-induced apoptosis in PC12 and SH-SY5Y cells ($P < 0.001$). These findings suggested that ^{99m}Tc-HYNIC-annexin V will be a promising radiochemical for the early detection of apoptosis in PD neurons.

There are some limitations of this study. Even though the binding of annexin V to PD cellular models was well correlated with the flow cytometry study, the question remains: Are the rates of apoptosis in this study comparable to the clinical situation in PD patients? Meanwhile, the uptake of annexin V by neurons is not specific for PD; apoptotic neurons in cases of ischaemic injury, inflammation, Alzheimer's disease and other disorders will also uptake annexin V. This is only an in-vitro binding study of ^{99m}Tc-HYNIC-annexin V to PD cellular models. The in-vivo imaging of PD animal models is currently being performed. It is generally accepted that PD dopaminergic nigral neurons die via apoptosis. Several human post-mortem studies have already proven that. However, the number of apoptotic neurons varies from 2 to 12%, with a large inter-individual variability [24,35–37]. Other studies have failed to detect apoptotic changes in PD patients [38,39]. This controversy may arise because it is difficult to preserve brain tissue after death, because of the relatively short half-life of apoptotic cells, or due to less sensitive methods for the detection of apoptosis. Considering that PD is a slowly progressive disease, the rate and number of cells undergoing apoptosis at a given time point is very low, so very sensitive methods are required for the early detection of apoptosis. However, for scintigraphic imaging with radiolabelled annexin V, the amount of neuronal death needed for detection has been unknown. Furthermore, imaging dopaminergic nigral neuron apoptosis in preclinical PD patients involves serious technical and theoretical problems, which make these studies difficult.

However, on the whole, ^{99m}Tc-HYNIC-annexin V is a promising radiochemical for the non-invasive detection of PD neuron apoptosis. If these observations play out in humans, the early diagnosis of PD is realistic, and the monitoring of disease progression or regression, or determining the efficacy of anti-apoptosis therapies, appears hopeful.

This in-vitro study provided evidence that ^{99m}Tc-HYNIC-annexin V retains its receptor-binding activity and has a high affinity to PD cellular models. The PD cellular uptake of radioactivity correlated well with cell apoptosis rates. ^{99m}Tc-HYNIC-annexin V is a promising radiopharmaceutical agent for in-vivo non-invasive imaging of early apoptosis in Parkinson's disease.

Acknowledgements

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