^{18}F-labelled annexin V: a PET tracer for apoptosis imaging

Yoshihiro Murakami[1], Hiroyuki Takamatsu[1, 2], Junichi Taki[2], Mitsuyoshi Tatsumi[1], Akihiro Noda[1, 2], Rikiya Ichise[1], Jonathan F. Tait[3], Shintaro Nishimura[1]

[1] The Medical and Pharmacological Research Center Foundation, Inoyama, Hakui, Ishikawa, Japan
[2] Department of Biotracer Medicine, Kanazawa University Graduate School of Medical Science, Kanazawa, Ishikawa, Japan
[3] Department of Laboratory Medicine, Medicine (Medical Genetics) and Pathology, University of Washington, Seattle, Washington, USA

Abstract. Annexin V can be used to detect apoptotic cells in vitro and in vivo, based on its ability to identify extracellular phosphatidylserine, which arises during apoptosis. In the present study, we examined the synthesis of fluorine-18 labelled annexin V as a positron emission tomography tracer for apoptosis imaging. The distribution of [^{18}F]annexin V and technetium-99m labelled annexin V, a well-characterised SPET tracer for apoptosis imaging, was compared. [^{18}F]annexin V was synthesised using *N*-succinimidyl 4-[^{18}F]fluorobenzoate as an ^{18}F labelling reagent. Synthesised and purified [^{18}F]annexin V was confirmed by SDS-PAGE. In an ex vivo imaging experiment, [^{18}F]annexin V was intravenously injected into rats 24 h after the induction of myocardial ischaemia, and accumulation in the left ventricle was examined. [^{18}F]annexin V accumulated in the infarct area of the left ventricle, where apoptotic cells were observed. In separate experiments, [^{18}F]annexin V or [^{99m}Tc]annexin V was intravenously injected into ischaemic or normal animals, and the distribution of the tracers was compared. In ischaemic animals, accumulation of [^{18}F]annexin V and [^{99m}Tc]annexin V in the infarct area was about three-fold higher than in the non-infarct area. Furthermore, the ratio of accumulation in the normal heart to the blood radioactivity was not significantly different between the tracers. In normal animals, however, the uptake of [^{18}F]annexin V in the liver, spleen and kidney was much lower than that of [^{99m}Tc]annexin V. The low uptake of [^{18}F]annexin V in these organs might represent an advantage over [^{99m}Tc]annexin V.

Keywords: Fluorine-18 – Annexin V – Apoptosis – Myocardial ischaemia – Positron emission tomography

Yoshihiro Murakami (✉)
The Medical and Pharmacological Research Center Foundation,
Wo32, 925-0613 Inoyama, Hakui, Ishikawa, Japan
e-mail: murakami@mprcf.or.jp
Tel.: +81-767-263311, Fax: +81-767-263314

Introduction

Annexin V is a 36-kDa calcium-dependent phospholipid binding protein [1] which has a high affinity for the membrane phospholipid phosphatidylserine (PS) [2, 3]. Since annexin V can be used to detect extracellular PS exposure [4] and PS exposure occurs during apoptosis [5], annexin V has been employed to detect apoptotic cells in vitro [6, 7] and in vivo [8].

On this basis, several in vivo studies on the imaging of apoptosis using annexin V labelled with technetium-99m or iodine-125 have been reported [9, 10, 11, 12, 13, 14]. However, the half-lives of ^{99m}Tc ($t_{1/2}$=6.02 h) and ^{125}I ($t_{1/2}$=60.2 days) are longer than the physiological apoptotic process, and labelling of annexin V with a positron emitter that has a relatively short half-life (e.g. ^{18}F, $t_{1/2}$=109.7 min) is required in order to examine the exact time course of apoptosis. In recent years, methods for the ^{18}F labelling of protein using ^{18}F-labelled reagents have been reported [15, 16, 17,18, 19, 20, 21, 22]. In these reports, *N*-succinimidyl 4-^{18}F-fluorobenzoate ([^{18}F]SFB) was found to be the most suitable reagent for ^{18}F labelling of protein. Therefore, we examined labelling of annexin V using [^{18}F]SFB as an ^{18}F labelling reagent.

In this study, we examined the synthesis of ^{18}F-labelled annexin V using a conventional method, and performed ex vivo imaging of apoptosis using a rat model of myocardial ischaemia-reperfusion. Furthermore, we compared the in vivo characteristics of [^{18}F]annexin V with those of [^{99m}Tc]annexin V, a well-characterised single-photon emission tomography (SPET) tracer for apoptosis imaging.

Materials and methods

Chemicals. Purified annexin V (100 μg in phosphate buffer, pH 7.4) was purchased from BD Pharmingen (San Jose, CA). Dimethyl sulphoxide, kryptofix2.2.2, 45% tetramethylammonium hydroxide and *O*-(*N*-succinimidyl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TSTU) were from Sigma-Aldrich Japan (Tokyo, Japan). 0.5 *M* NaOH, 0.5 *M* HCl, acetonitrile, methanol, sodium phosphate dibasic, potassium phosphate monobasic and phosphate buffered saline were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Ethyl 4-trimethylammoniumbenzoate triflate was synthesised according to the literature [23].

Synthesis of ^{18}F-labelled N-succinimidyl 4-fluorobenzoate. The title compound was prepared as described by Wester et al. [22] with modifications. Production of ^{18}F$^-$ was accomplished via an ^{18}O(p, n)^{18}F reaction by proton bombardment (12 MeV, 50 μA) of an ^{18}O-water target using a cyclotron-target system (OSCAR-12, JFE Plant & Service Corporation, Yokohama, Japan). After irradiation, the ^{18}F-containing target water was transferred to an ^{18}F recovery system. The target material was passed through an anion exchange resin (SepPak QMA, Nihon Waters K.K., Tokyo, Japan) and recovered as [^{18}F]KF by elution with aqueous potassium carbonate (1.0 ml, 20 m*M*) into a conical glass vial. To this solution, kryptofix 2.2.2. (20 mg, 53 μmol) in acetonitrile was added. The recovered ^{18}F$^-$ solution was then transferred to the glass vessel in the synthesis apparatus using He gas. The aqueous solution was removed azeotropically with acetonitrile at 120°C. This operation was repeated three times. To the residue a solution of the starting material [ethyl 4-trimethylammoniumbenzoate triflate (10.0 mg, 52 μmol)] in DMSO (0.6 ml) was added. The mixture was heated at 110°C for 15 min. After the reaction mixture was cooled to room temperature, 0.5 *M* NaOH (0.5 ml) was added. The mixture was heated at 80°C for 5 min, and then neutralised with 0.5 *M* HCl (0.6 ml). The mixed solution was loaded onto a high-performance liquid chromatography (HPLC) column and eluted with phosphate buffer pH 6.0: CH_3CN=85:15 (flow rate: 4.3 ml/min; column: YMC-Pack C18 Pro 10×250 mm, 10 μm, YMC Co., Ltd, Kyoto, Japan) to give a fraction containing 4-[^{18}F]fluorobenzoic acid, which was collected and the solvent removed under reduced pressure. To the residue was added a solution of 45% tetramethylammonium hydroxide (40 μl) in water (0.2 ml) and CH_3CN (1.0 ml), evaporated by an N_2 flush and reduced pressure. After evaporation, TSTU (14 mg) in CH_3CN (1.2 ml) was added to the residue, and then it was heated at 90°C for 2 min. After reaction, 1% AcOH (8 ml) was added to the reaction mixture. The mixed solution was passed through a SepPak Plus C18 (Nihon Waters K.K.), and washed with 30% CH_3CN (8 ml). Finally, the product was eluted with CH_3CN (2.0 ml), and then evaporated by an N_2 flush. Analytical chromatography was performed with the Agilent 1100 HPLC system (Agilent Technologies Japan, Tokyo) and an Aloka positron detector RLC-700 (Aloka Co., Ltd, Tokyo, Japan) using a Finepak SIL C18S column (5 μm, 4.6×150 μm, JASCO Corporation, Tokyo, Japan), eluted with 0.1% AcOH-0.05 *M* NH$_4$OAc: CH_3CN=50:50 at a flow rate of 1 ml/min.

Synthesis of [^{18}F]annexin V. To the residue of [^{18}F]SFB (1.5–2 GBq), an annexin V (100 μg) solution of 0.5 *M* borate buffer pH 9.2 (50 μl) was added and reacted at room temperature for 15 min. The reaction mixture was purified with a size-exclusion column chromatography procedure, as follows: Bio-Gel P-6DG gel (Bio-Rad Japan, Tokyo) was fitted to a glass column (25 mm inner diameter × 50 mm). The reaction mixture was diluted with saline (1 ml) and applied to the column. The component was eluted with saline to give a fraction containing [^{18}F]annexin V (90–160 MBq) after the void volume of about 6 ml.

SDS-PAGE. SDS-PAGE was performed with a Mini-PROTEIN and POWER PAC 300 (Bio-Rad, Hercules, CA). Laemmli sample buffer (Bio-Rad, Hercules, CA) was added to each sample (1:1) and applied to Readygels J (Bio-Rad, Hercules, CA). Precision Protein Standard (Bio-Rad, Hercules, CA) was used as a standard. Electrophoresis was performed under constant voltage (200 V). After electrophoresis, gels were exposed to an imaging plate and radioactivity was analysed by a Fuji Bass system (Bass 1800, Fuji Film, Tokyo, Japan), followed by staining with Bio-Safe Coomassie (Bio-Rad, Hercules, CA).

Synthesis of [^{99m}Tc]annexin V. Mutant annexin V (annexin V-117 mutant) was prepared through expression in *Escherichia coli* as previously described [9]. This material retains PS binding activity equivalent to that of native annexin V. A specific activity of 3.7–7.4 MBq (100–200 μCi)/μg protein with a radiopurity of more than 90% was achieved using the previously described radiolabelling protocol [9].

Animal preparation. The studies were performed on male Wistar rats with body weights ranging from 198 to 273 g (Japan SLC, Shizuoka, Japan). All experiments were performed in accordance with the institutional guidelines of The Medical and Pharmacological Research Center Foundation.

Animals were anaesthetised with sodium pentobarbital (30 mg/kg i.p.), tracheostomised and artificially ventilated (140 ml/min, 40 beats/min) with a small animal ventilator (CIV-101, Columbus Instruments, Columbus, OH). The chest was opened between the fourth and the fifth ribs, and the coronal artery was ligated using a 4-0 nylon suture. For the ischaemia-reperfusion model, 20 min after ligation the suture was removed and the incision was closed. An electrocardiogram was also monitored during surgery. The body temperature of each animal was maintained at 37.5 °C using a heat pad (NS-TC10, Neuroscience Inc., Osaka, Japan).

Ex vivo imaging for [^{18}F]annexin V. Twenty-four hours after myocardial ischaemia, [^{18}F]annexin V (15 MBq, 10 μg) was intravenously administered. One hour after the injection, the heart was removed under sodium pentobarbital anaesthesia, the left ventricular was sectioned, and three preselected coronal sections (at 2-mm intervals) were made. Each section was stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC) in saline. After photographs of each section had been taken, the area of myocardial infarction was measured using a computerised imaging system (Win ROOF, Mitani Corporation, Fukui, Japan), and the ratio of infarction area to the whole left ventricular area was calculated. Then, each section was exposed to an imaging plate (Fuji Film, Tokyo, Japan) for 10 min. Radioactivity was converted into digitalised imaging data via a Fuji Bass system (Bass 1800, Fuji Film, Tokyo, Japan).

Comparison of distribution of [^{18}F]annexin V and [^{99m}Tc]annexin V. [^{18}F]annexin V (15 MBq, 10 μg) or [^{99m}Tc]annexin V (15 MBq, 3 μg) was intravenously injected into the animals with myocardial ischaemia (24 h after ischaemia) or normal animals. Animals were decapitated under pentobarbital deep anaesthesia, and blood samples were collected. The heart, liver, spleen and kidney were removed. In the rats with cardiac ischaemia, three preselected coronal (short axial) sections (at 2-mm intervals) were made, and each section was stained with 1% TTC. Then, infarcted and normal ar-

Fig. 1. Synthetic route for [18F]-labelled annexin V. TSTU, *O-(N-succinimidyl)-N,N,N',N'-tetramethyluronium tetrafluoroborate*
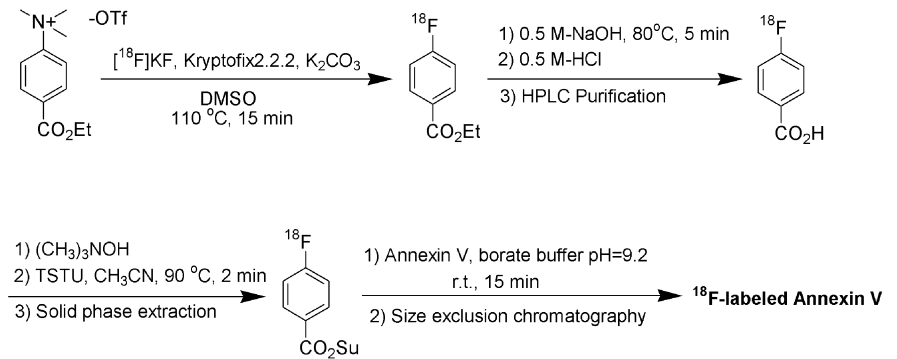
eas were separated. These tissues and blood samples were weighed, and their radioactivity was measured using an auto well gamma counter (1480 WIZARD; Wallac Oy, Turku, Finland). The ratio of the radioactivity of each tissue (cpm/mg) to the blood radioactivity (/cpm/mg) was calculated.

Pathological studies. In a separate experiment, the area of ischaemic damage and apoptotic cells following myocardial ischaemia-reperfusion were examined using three animals. Twenty-four hours after ischaemia, the left ventricles were sectioned into serial short axial slices and fixed in a 10% formalin neutral buffer solution, pH 7.4 for 2 days, and three preselected coronal (short axial) sections (at 2-mm intervals) were made. Each section was embedded in paraffin wax, and 10-μm-thick sections were cut and stained with haematoxylin and eosin (HE) and with an apoptosis detection kit (Apop Tag-Plus, Intergen Company, NY).
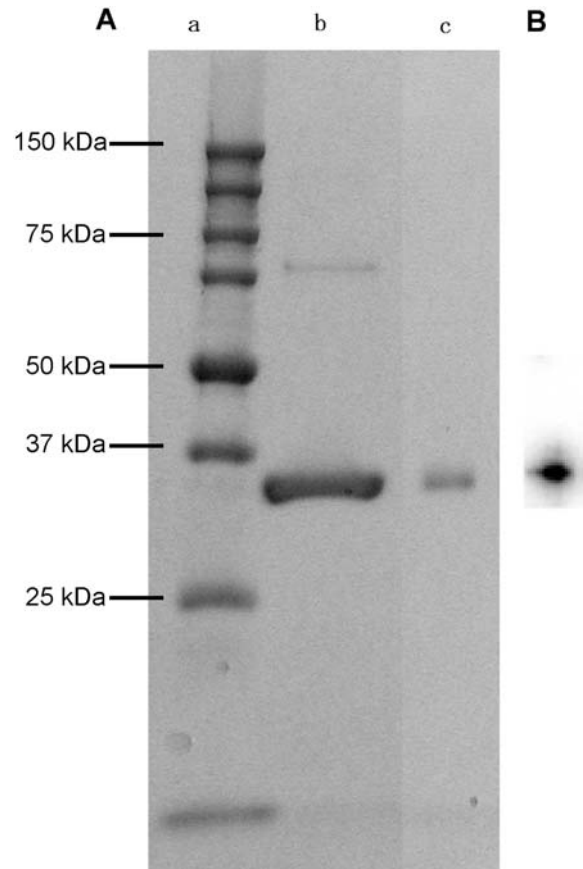
Results

Synthesis of [18F]annexin V

The synthetic route for [18F]annexin V is shown in Fig. 1. The decay-corrected radiochemical yield of [18F]SFB was 30–40% based on 18F-fluoride, and radiochemical purity was higher than 98%. The preparation time was about 1 h. Concerning the conjugation with annexin V, the reaction was performed under a basic condition. A borate buffer 0.5 M solution, pH 9.2 was used for the conjugation, and the concentration was increased as much as possible. The reaction with [18F]SFB was performed at room temperature for 15 min, and purified to give [18F]annexin V in about a 10% yield (decay corrected) from [18F]SFB.

SDS-PAGE

Fig. 2A shows the result of Coomassie staining of polyacrylamide gels after electrophoresis of standard annexin V, purified [18F]annexin V and a molecular weight standard. Annexin V was not affected by any degradation during [18F]annexin V synthesis; [18F]annexin V was successfully purified, and the radiochemical purity was >99% (Fig. 2B).


Fig. 2A, B. SDS-PAGE analysis of purified [18F]annexin V. The result of the Coomassie stain is shown in **A**. *Lane a*, molecular weight standard; *lane b*, standard annexin V; *lane c*, purified [18F]annexin V. After electrophoresis, gels were exposed to an imaging plate and radioactivity was analysed (**B**)

Detection of apoptosis following myocardial ischaemia

Twenty-four hours after myocardial ischaemia-reperfusion, the size of myocardial infarction of the left ventricle was 46.6%±9.3% (*n*=3) on the TTC stain. The area of myocardial infarction was not different between the TTC and HE stains (data not shown). Apoptosis was widely observed only in the infarct area of the left ventricle (Fig. 3).

472

Fig. 3A–C. Typical results of an HE stain of the left ventricle 24 h after ischaemia (**A**), and detection of apoptosis in the regions identified in **A** (**B, C**)
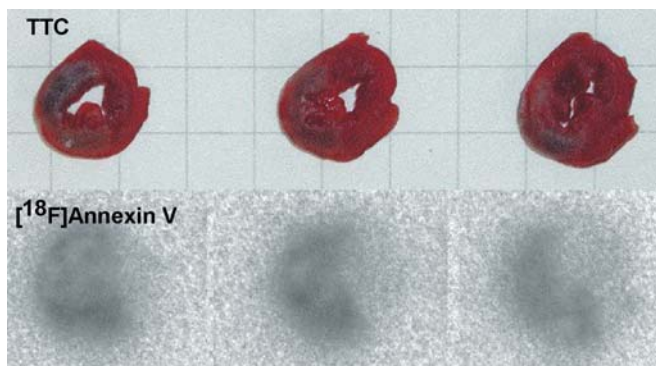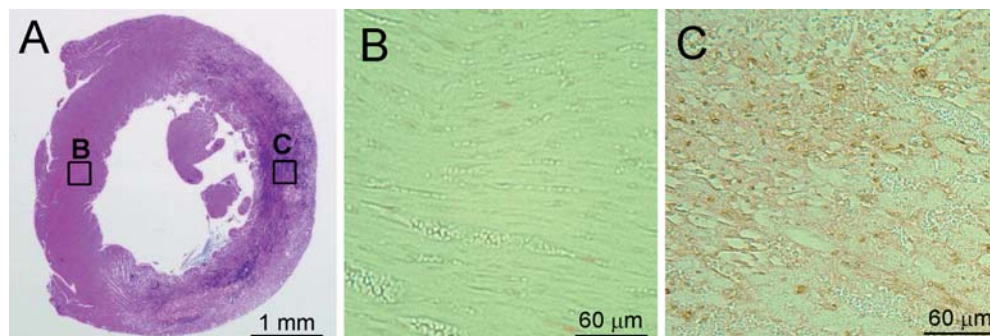
Fig. 4. Typical infarction in the left ventricle 24 h after ischaemia (TTC stain) and accumulation of [18F]annexin V

Fig. 5. Accumulation of [18F]annexin V or [99mTc]annexin V in the infarction of the left ventricle 24 h after ischaemia. Each *column* represents the mean ratio of annexin V accumulation in infarction to that in non-infarction in three animals, and the *bars* represents SD
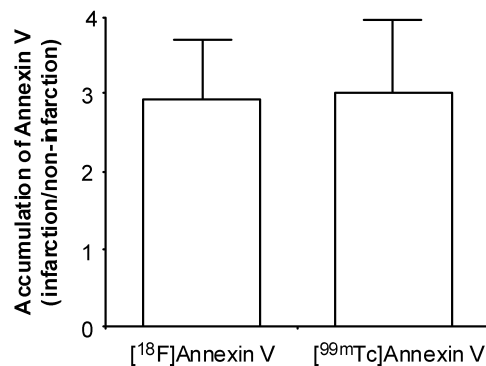
Ex vivo imaging for [18F]annexin V

Figure 4 shows the left ventricular infarct area 24 h after ischaemia, stained by TTC, and the exposure image of the same slice. The accumulation of [18F]annexin V in the left ventricular infarct area 24 h after ischaemia matched the infarct area detected by TTC staining.
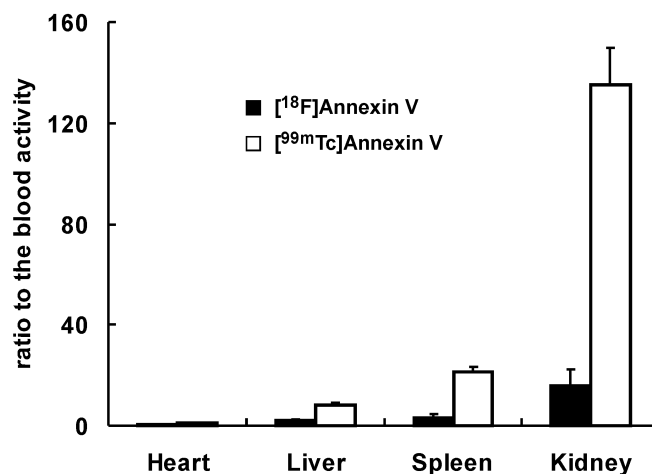
Comparison of distribution of [18F]annexin V and [99mTc]annexin V

In ischaemic animals, accumulation of [18F]annexin V and [99mTc]annexin V in the infarct area showed similar increases, by 2.9±0.8 and 3.0±0.9 fold respectively, compared with the non-infarct area (Fig. 5).

Accumulation of [18F]annexin V and [99mTc]annexin V in the heart, liver, spleen and kidney in normal animals is shown in Fig. 6. The ratio of accumulation in the normal heart to the blood activity was not significantly different between the tracers ([18F]annexin V, 0.7±0.2; [99mTc]annexin V, 0.9±0.1). However, accumulation of [99mTc]annexin V was significantly higher than that of [18F]annexin V in the liver ([18F]annexin V, 2.1±0.5; [99mTc]annexin V, 8.3±0.8), spleen ([18F]annexin V, 3.0±1.5; [99mTc]annexin V, 21.6±1.9) and kidney ([18F]annexin V, 15.8±6.8; [99mTc]annexin V, 135.3±14.6).

Fig. 6. Accumulation of [18F]annexin V or [99mTc]annexin V in the heart, liver, spleen and kidney in normal rats. The *closed columns* represent the accumulation of [18F]annexin V, and the *open columns* represents that of [99mTc]annexin V. Each *column* represents the mean ratio of annexin V accumulation in each tissue to the blood activity in three animals, and the *bars* represents SD

Discussion

In the present study, [18F]annexin V was successfully synthesised using [18F]SFB as an 18F labelling reagent. After size-exclusion column chromatography, [18F]annexin V could be purified without any degradation.

Several animal studies have shown that apoptosis can be detected histopathologically in acute myocardial infarction [24, 25, 26]. Furthermore, in vivo imaging of apoptosis using [99mTc]annexin V has been demonstrated in an animal model [27] and human myocardial infarction [28]. Therefore, in order to examine detection of apoptosis, an ex vivo imaging experiment was performed in a rat model of myocardial ischaemia and reperfusion. First, we examined the characteristics of the model. In this model, reproducible myocardial infarction was constantly produced in the left ventricle 24 h after ischaemia, as has been reported previously [29, 30, 31]. Apoptotic cells were widely detected only in the infarct area stained by HE. Therefore, in further experiments, we used a TTC stain to detect infarction as a convenient conventional method [29, 30, 31].

The ex vivo imaging experiment revealed that accumulation of [18F]annexin V was observed in the infarct area of the left ventricle 24 h after ischaemia. This result suggested that [18F]annexin V accumulated in the apoptotic cells, and demonstrated that [18F]annexin V could detect apoptosis. In order to examine the ability of [18F]annexin V to detect apoptosis, we compared the accumulation in the infarct area of [18F]annexin V and [99mTc]annexin V, a well-characterised SPET tracer for the detection of apoptosis [9, 10, 11, 12, 13, 14]. Accumulation of [18F]annexin V and [99mTc]annexin V in the infarction was almost the same, with about a threefold increase in comparison with the non-infarct area. This result indicates that the ability of [18F]annexin V to detect apoptosis in myocardial ischaemia is equal to that of [99mTc]annexin V. Therefore, this is the first report on synthesis of a PET tracer for apoptosis imaging.

One difference between [18F]annexin V and [99mTc]annexin V was their distribution in the liver, spleen and kidney. In normal animals, the ratios of [99mTc]annexin V accumulation in the liver, spleen and kidney to the blood radioactivity were significantly higher than those obtained for [18F]annexin V. These results indicate that the uptake of [99mTc]annexin V in these tissues is significantly higher than that of [18F]annexin V. This might be due to the characteristics of 99mTc as a heavy metal. The low uptake characteristics of [18F]annexin V in these tissues could be one advantage over [99mTc]annexin V because of the physiological and pharmacological convenience, for example in the assessment of tumour response in the abdomen early after chemotherapy [32].

In conclusion, in this study we successfully synthesised and purified [18F]annexin V as the first PET tracer for apoptosis imaging. The ability of [18F]annexin V to detect apoptosis was comparable to that of [99mTc]annexin V. The low uptake of [18F]annexin V in the liver, spleen and kidney could offer an advantage over [99mTc]annexin V.

References

1. Raynal P, Pollard HB. Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. *Biochim Biophys Acta* 1994; 1197: 63–93.

2. Tait JF, Gibson D, Fujikawa K. Phospholipid binding properties of human placental anticoagulant protein-I, a member of the lipocortin family. *J Biol Chem* 1989; 264: 7944–7949.

3. Andree HA, Reutelingsperger CP, Hauptmann R, Hemker HC, Hermens WT, Willems GM. Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers. *J Biol Chem* 1990; 265:4923–4928.

4. Thiagarajan P, Tait JF. Binding of annexin V/placental anticoagulant protein I to platelets. Evidence for phosphatidylserine exposure in the procoagulant response of activated platelets. *J Biol Chem* 1990; 265:17420–17423.

5. Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol* 1992; 148:2207–2216.

6. Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* 1994; 84:1415–1420.

7. Martin SJ, Reutelingsperger CP, McGahon AJ, et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* 1995; 182:1545–1556.

8. Blankenberg FG, Katsikis PD, Tait JF, et al. In vivo detection and imaging of phosphatidylserine expression during programmed cell death. *Proc Natl Acad Sci USA* 1998; 95: 6349–6354.

9. Tait JF, Brown DS, Gibson DF, Blankenberg FG, Strauss HW. Development and characterization of annexin V mutants with endogenous chelation sites for (99m)Tc. *Bioconjug Chem* 2000; 11:918–925.

10. Blankenberg FG, Katsikis PD, Tait JF, et al. In vivo detection and imaging of phosphatidylserine expression during programmed cell death. *Proc Natl Acad Sci USA* 1998; 95: 6349–6354.

11. Blankenberg FG, Katsikis PD, Tait JF, et al. Imaging of apoptosis (programmed cell death) with 99mTc annexin V. *J Nucl Med* 1999; 40:184–191.

12. Blankenberg FG, Robbins RC, Stoot JH, et al. Radionuclide imaging of acute lung transplant rejection with annexin V. *Chest* 2000; 117:834–840.

13. Russell J, O'Donoghue JA, Finn R, et al. Iodination of annexin V for imaging apoptosis. *J Nucl Med* 2002; 43:671–677.

14. Vriens PW, Blankenberg FG, Stoot JH, et al. The use of technetium Tc 99m annexin V for in vivo imaging of apoptosis

474

during cardiac allograft rejection. *J Thorac Cardiovasc Surg* 1998; 116:844–853.

15. Garg PK, Garg S, Zalutsky MR. Fluorine-18 labeling of monoclonal antibodies and fragments with preservation of immunoreactivity. *Bioconjug Chem* 1991; 2:44–49.

16. Guhlke S, Coenen HH, Stocklin G. Fluoroacylation agents based on small n.c.a. [18F]fluorocarboxylic acids. *Appl Radiat Isot* 1994; 45:715–727.

17. Herman LW, Fischman AJ, Tompkins RG, et al. The use of pentafluorophenyl derivatives for the 18F labelling of proteins. *Nucl Med Biol* 1994; 21:1005–1010.

18. Kilbourn MR, Dence CS, Welch MJ, Mathias CJ. Fluorine-18 labeling of proteins. *J Nucl Med* 1987; 28:462–470.

19. Lang L, Eckelman WC. One-step synthesis of 18F labeled [18F]-*N*-succinimidyl 4-(fluoromethyl)benzoate for protein labeling. *Appl Radiat Isot* 1994; 45:1155–1163.

20. Shai Y, Kirk KL, Channing MA, et al. 18F-labeled insulin: a prosthetic group methodology for incorporation of a positron emitter into peptides and proteins. *Biochemistry* 1989; 28:4801–4806.

21. Vaidyanathan G, Zalutsky MR. Labeling proteins with fluorine-18 using *N*-succinimidyl 4-[18F]fluorobenzoate. *Int J Rad Appl Instrum B* 1992; 19:275–281.

22. Wester HJ, Hamacher K, Stocklin G. A comparative study of N.C.A. fluorine-18 labeling of proteins via acylation and photochemical conjugation. *Nucl Med Biol* 1996; 23:365–372.

23. Haka MS, Kilbourn MR, Watkins GL, Toorongian SA. Aryl-trimethylammonium trifluoromethanesulfonates as precursor to aryl [18F]fluorides: improved synthesis of [18F]GBR-13119. *J. Label Compd Radiopharm* 1988; 27:823–833.

24. Kajstura J, Cheng W, Reiss K. Apoptotic and necrotic myocyte cell deaths are independent contributing variables in infarct size in rats. *Lab Invest* 1996; 74:86–107.

25. Fliss H, Gattinger D. Apoptosis in ischemic and reperfused rat myocardium. *Circ Res* 1996; 79:949–956.

26. Bialik S, Geenen DL, Sasson IE, et al. Myocyte apoptosis during acute myocardial infarction in the mouse localizes to hypoxic regions but occurs independently of p53. *J Clin Invest* 1997; 100:1363–1372.

27. Dumont EA, Hofstra L, van Heerde WL, et al. Cardiomyocyte death induced by myocardial ischemia and reperfusion: measurement with recombinant human annexin-V in a mouse model. *Circulation* 2000; 102:1564–1568.

28. Hofstra L, Liem IH, Dumont EA, et al. Visualisation of cell death in vivo in patients with acute myocardial infarction. *Lancet* 2000; 356:209–212.

29. Albertini A, Zucchini P, Noera G, Cadossi R, Napoleone CP, Pierangeli A. Protective effect of low frequency low energy pulsing electromagnetic fields on acute experimental myocardial infarcts in rats. *Bioelectromagnetics* 1999; 20:372–377.

30. Aye NN, Komori S, Hashimoto K. Effects and interaction of cariporide and preconditioning on cardiac arrhythmias and infarction in rat in vivo. *Br J Pharmacol* 1999; 127:1048–1055.

31. Bernauer W. The effect of beta-adrenoceptor blocking agents on evolving myocardial necrosis in coronary ligated rats with and without reperfusion. *Naunyn Schmiedebergs Arch Pharmacol* 1985; 328:288–294.

32. Belhocine T, Steinmetz N, Hustinx R, et al. Increased uptake of the apoptosis-imaging agent (99m)Tc recombinant human annexin V in human tumors after one course of chemotherapy as a predictor of tumor response and patient prognosis. *Clin Cancer Res* 2002; 8:2766–2774.