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SYNTHESIS AND IN VITRO STABILITY OF ^{123}I-LABELLED ANNEXIN V : A POTENTIAL AGENT FOR SPECT IMAGING OF APOPTOTIC CELLS

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SUMMARY

Human recombinant annexin V was radiolabelled with iodine-123 by electrophilic substitution of the containing tyrosine residues using carrier-free sodium iodide-123 as the isotope source. The synthesis of ^{123}I-labelled annexin V was performed using IodoBeads as oxidative solid phase iodinating agent and reaction parameters were optimized and evaluated. Purification by straight-phase HPLC gave [^{123}I]-annexin V in a maximum yield of approximately 70 % with a radiochemical purity of more than 95 % and a specific activity of about 10 MBq / μg of protein. Furthermore, in vitro stability experiments indicated that [^{123}I]-annexin V was stable for at least 8 hours in an aqueous phosphate buffer solution. In plasma however it decreased more rapidly. The biological activity of [^{123}I]-annexin V remained intact in comparison with FITC labelled annexin V as confirmed by in vitro binding experiments with apoptotic cells.

Key words: Annexin V, ^{123}I-labelling, Apoptosis

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INTRODUCTION

Annexin V is a Ca^{2+}-dependent 36 kD protein that shows high affinity for phosphatidylserine-containing membranes. Apoptosis (= programmed cell death) plays an important role in several biological processes like embryogenesis, homeostasis and regulation of the immune system (1-3). One of the main events in the early stage of apoptosis is the disturbance of the normal cell membrane phosphatidylserine asymmetry. Phosphatidylserine-residues (PS) in blood platelets, erythrocytes and healthy human cells are normally located on the inner leaflet of the cell membrane (4). In activated blood platelets, sickle-cell erythrocytes and in cancer cells undergoing apoptosis, these phosphatidylserine-residues are translocated to the outer leaflet of the cell membrane thereby exposing PS to the extracellular environment (5-6). As this phenomenon of membrane blebbing is seen as one of the first stages in the apoptosis process, ^{123}I-labelled annexin V can be a sensitive SPECT-ligand for the early detection of cancer cells, infarcted myocardium 'hot spots' and thrombi in nuclear medicine. In this paper the optimum labelling conditions as well as the in vitro stability results are presented.

MATERIALS AND METHODS

Materials

Materials were obtained from the following sources: Iodo-Beads – Pierce Biochemical Co., Rockford, Illinois; $Na^{123}I$ – Nycomed Amersham, Brussels, Belgium; human recombinant annexin V – Becton Dickinson, Erembodegem, Belgium; FITC labelled annexin V, BenderMed Systems, Vienna, Austria; bovine serum albumin – Sigma-Aldrich, Steinheim, Germany. All other chemicals, reagent grade or better, were from Sigma-Aldrich.

Optimisation of [^{123}I]-annexin V radiosynthesis

Synthesis of ^{123}I-labelled annexin V was performed according to the Iodo-Bead method (7-8). The Iodo-Bead method applies chloramine-T molecules immobilized

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on nonporous polystyrene beads as solid phase iodination agent. Prior to use, Iodo-Beads were washed systematically in a 0.01 M KH_2PO_4 (pH 7.4) solution and dried on adsorbent paper. The iodination reaction was initiated by adding one bead to a mixture of 0.75 MBq carrier free $Na^{123}I$ (dissolved in 60 μL 0,05 M NaOH), 5 μg annexin V (dissolved in 10 μL PBS pH 7.4) and 50 μL (0.1 M) KH_2PO_4 buffer (pH 6.5). On completion, iodination was terminated by simply removing the Iodo-Bead from the tube.

During optimisation of the ^{123}I-labelling procedure, radiolabelling efficiencies were determined by TCA-precipitation as follows: The precipitation reaction was carried out for 30 min on ice by adding 10 μL (2.5 %) BSA and 900 μL (7.5 %) trichloroacetic acid to a 10 μL aliquot of the reaction mixture. After centrifugation at 1500 g for 10 min at room temperature (18°C), the supernatant was removed and the pellet was washed twice with 1 mL (7.5 %) TCA. Finally, the radioactivity of pellet and all supernatants was measured on a LKB γ-counter and corrected for background.

Determination of the radiochemical yield as a function of reaction time

Influence of the reaction time on the labelling yield was determined by adding one Iodo-Bead to a mixture of 0.75 MBq $Na^{123}I$, 5 μg annexin V and 50 μL (0.1 M) KH_2PO_4 buffer (pH 7). Reaction was allowed to proceed for several time intervals at room temperature (18°C) and radiochemical yields were determined by TCA-precipitation.

Determination of the radiochemical yield as a function of pH

The effect of the reaction mixture pH on the labelling yield was investigated by varying the pH of the reaction buffer. Therefore, three different buffers (i.e. citric acid, potassium phosphate and sodium borate) were applied over a different pH range with regard to their buffer capacity. Iodination was carried out for 5 min at 18°C using 0.75 MBq $Na^{123}I$, 5 μg annexin V and 50 μL (0.1 M) reaction buffer. Subsequently radiochemical yields were determined by TCA-precipitation.

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Determination of the radiochemical yield as a function of buffer concentration

As the KH_2PO_4 buffer (pH 6.5) proved to be most suitable for iodination of the tyrosine residues of annexin V, radiochemical yields were determined as a function of different KH_2PO_4 buffer concentrations at pH 6.5. All other reaction parameters were applied as described above and radiochemical yields were determined by TCA-precipitation.

Determination of the radiochemical yield as a function of temperature

Measurement of the labeling yield as a function of reaction temperature was accomplished by adding one bead to a mixture of 0.75 MBq $Na^{123}I$, 5 μg annexin V and 50 μL, 0.1 M KH_2PO_4 buffer (pH 6.5). After 5 min at a given temperature, the reaction was terminated and the labelling efficiency was measured by means of TCA-precipitation.

Determination of the radiochemical yield as a function of the Iodo-Beads amount

The use of multiple Iodo-Beads in one iodination reaction and its effect on the radiochemical yield was also determined in the optimisation experiments. The same reaction conditions were used as mentioned above and the reaction was carried out at room temperature.

HPLC conditions for [^{123}I]-annexin V purification

In order to investigate the in vitro stability of [^{123}I]-annexin V, a straight-phase HPLC system was developed for the separation of radiolabelled protein from remaining unreacted ^{123}I. The HPLC set up consisted of a 6-valve injector with 100 μL loop, a Waters 510 HPLC pump, a LC-UV detector (Pye Unicam) at 254 nm, a Ludlum 2200 NaI (Tl) gamma detector and two one-channel C-R6A chromatopac recorders (Shimadzu). Chromatographic separation was performed on a Waters 120 Å Ultra-hydrogel column (7.8 × 300 mm) using 0.01 M KH_2PO_4 as eluent at a flow rate of 0.8 mL/min. Radiochemical yields as determined by the Iodo-Bead method under optimized reaction conditions, were also confirmed by this HPLC-procedure.

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In vitro stability of [^{123}I]-annexin V

Two kinds of experiments were performed to determine the stability in vitro. In the first method [^{123}I]-annexin V obtained by HPLC-purification as described above, was allowed to stand at room temperature (18 °C) for 27 hours. After 10 and 30 min and 1, 3, 6, 8, 12, 24 and 27 hour, aliquots of the tracer were reinjected on the same HPLC-system and checked for radiochemical purity.

In the second method duplicate samples of [^{123}I]-annexin V were incubated at 37°C in plasma from healthy NMRI mice. Therefore blood was drawn from NMRI mice, collected in EDTA tubes and centrifuged at 1000 g for 20 min. Afterwards the plasma was removed from the tubes and stored at 37°C for incubation. During the incubation 10 µL fractions were taken after 5, 10, 15, 30, 60 and 120 min time intervals and spotted on silica gel TLC-plates (Macherey-Nagel GmbH & Co., Düren) using 0.01 M KH_2PO_4 (pH 7.4) as eluent. After thin-layer chromatography, the plates were removed from the chamber, cut into 4 mm sections and counted for radioactivity in a LKB 1260 multigamma-counter.

In vitro biological activity of [^{123}I]-annexin V

In order to investigate if annexin V remained its biological activity after iodination, an in vitro binding assay of [^{123}I]-annexin V with apoptotic cells was performed and compared with FITC labelled annexin V. Therefore peripheral blood lymphocytes (PBL) from healthy volunteers were induced to undergo apoptosis by means of actinomycin D, cycloheximide, γ-irradiation (5 Gy) and five different synthetic analogues of ceramide. The isolation and treatment of the cells was carried out according to a method described previously (9). Twenty-four hours after apoptosis induction all cell samples were divided into two parts and incubated with [^{123}I]-annexin V or FITC labelled annexin V, respectively. Cells without any treatment and treated cells in the absence of [Ca^{2+}] were used as controls.

One part of the treated cells (0.75 10^6 cells/mL) was incubated with 0.5 µg/mL [^{123}I]-annexin V in the presence of 5 mM [Ca^{2+}] (final concentration) for 1 hour at room temperature. After centrifugtion of the samples for 5 min at 800 g the pellet was washed once with 1 ml ice cold HEPES buffer (10 mM HEPES, 0.14 mM

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NaCl, 2.5 mM $CaCl_2$, NaOH pH 7.4) and again centrifuged. Finally, the radioactivity of pellet and supernatans were counted on a NaI γ-counter in order to compare the percentage of cell bound [^{123}I]-annexin V with the percentage of apoptosis as determined by flow cytometry (Table 4).

The second part of the treated cells was used for the flow cytometry assay. Staining of the cells was performed as described before (9). Briefly, the cells were resuspended in 50 µL of HEPES buffer (10 mM HEPES-NaOH, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.4) and incubated with 1 µL of FITC labelled annexin V for 15 min (room temperature, dark). Afterwards, 500 µL of HEPES buffer was added and the samples were analyzed (9). Flow cytometry was performed on a FACSort (Becton Dickinson) equipped with a single argon ion laser. A minimum of 10,000 cells were analyzed per sample and data analysis was performed with CellQuest software (BD).

RESULTS AND DISCUSSION

Optimisation of the [^{123}I]-annexin V radiosynthesis

During optimisation of the radiosynthesis, following reaction parameters were evaluated: reaction time, pH of reaction mixture, potassium phosphate buffer concentration, reaction temperature and number of Iodo-Beads added to the vial. Optimisation was systematically performed by varying one parameter at a time while keeping the other parameters constant. All experiments were performed in threefold.

Radiochemical yield as a function of reaction time

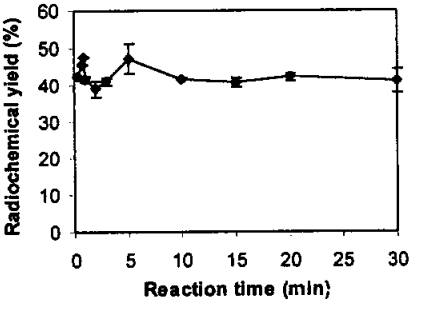

Figure 1. Radiochemical yield as a function of reaction time

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The radiochemical yield reached a maximum (47.1 %) at a reaction time of 5 min and showed a slight decrease at 10 min in order to stay constant for all longer reaction times. Although a first time optimum (47.5 %) seemed to occur at 50 sec, all further iodination reactions were preferentially performed with a reaction time of 5 min.

Radiochemical yield as a function of pH

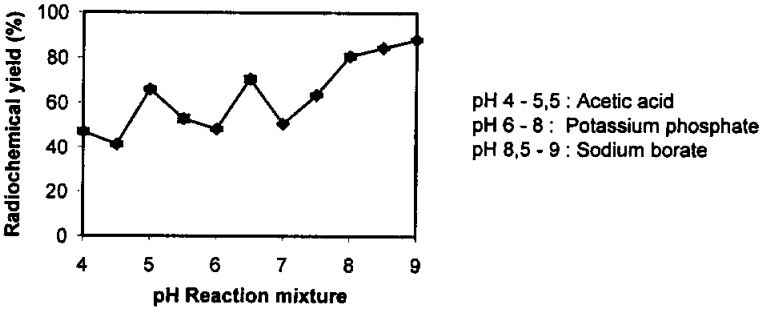

Figure 2. Radiochemical yield as a function of reaction mixture pH

As shown in Figure 2, three maxima in radiochemical yield can be observed at respectively pH 5 (66.0 %), pH 6.5 (70.6 %) and pH 9 (88.2 %). The maximum at pH 5 is probably due to the fact that apart from tyrosine, the cysteïne residue in annexin V can also become iodinated at lower pH levels whereas the maximum at pH 9 can be explained by concomitant iodination of histidine residues. As iodination of mainly tyrosine residues in the protein is most likely in the neutral pH range, we decided to continue the optimisation of the [^{123}I]-annexin V synthesis at the pH 6.5 optimum.

Radiochemical yield as a function of KH_2PO_4-concentration

KH_2PO_4-concentration (M)	Radiochemical yield (%)
0.01	68.3 ± 0.3
0.1	70.6 ± 1.2
1	68.6 ± 1.2

Table 1. Radiochemical yield as a function of KH_2PO_4-concentration

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Concerning the effect of the molarity of reaction buffer on the radiochemical yield of the iodination reaction, we observed no significant influence of this reaction parameter. Further optimisation experiments were performed using the 0.1 M KH_2PO_4 buffer (pH 6.5).

Radiochemical yield as a function of reaction temperature

Reaction temperature (°C)	Radiochemical yield (%)
0	64.4 ± 1.9
18	70.6 ± 1.2
37	61.2 ± 2.4

Table 2. Radiochemical yield as a function of reaction temperature

As high temperatures are known to be critical concerning the structural features and the related biological activity of proteins, the reaction temperature was varied to a maximum of 37°C. According to the results in Table 2, ^{123}I-labelling of annexin V seemed slighly favoured at room temperature (18°). Therefore all other experiments were carried out at 18°C.

Radiochemical yield as a function of number of Iodo-Beads

Number of Iodo-Beads	Radiochemical yield (%)
1	70.6 ± 1.2
2	82.2 ± 0.1
3	85.7 ± 0.1

Table 3. Radiochemical yield as a function of number of Iodo-Beads

In the experiments in which multiple Iodo-Beads were used for the iodination reaction, the obtained radiochemical yields showed a significant increase when two beads were applied instead of one. Additional use of a third Iodo-Bead did not result in a further substantial increase. However, the main drawback for application of multiple beads in one reaction is the increased adsorption of [^{123}I]-annexin V to

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the polystyrene beads. As we noticed, approximately 6 % of radiolabelled annexin V was adsorbed on each Iodo-Bead after reaction. These results indicate that a compromise has to be made between a higher radiolabelling efficiency and a lower chemical yield when multiple Iodo-Beads are used during iodination. Finally it was decided to continue all further [^{123}I]-annexin V productions with one Iodo-Bead.

In vitro stability

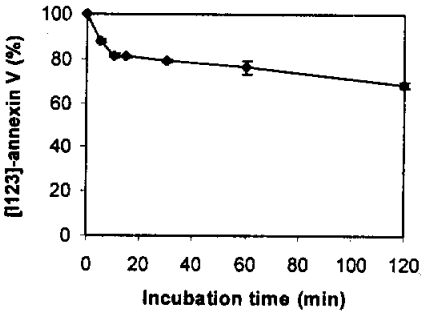

Figure 3. Radiochemical purity of [^{123}I]-annexin V in plasma as a function of time

The in vitro stability studies in aqueous phosphate buffer solution demonstrated that 98 % of ^{123}I-labelled annexin V was found intact after a period of 8 hours. In plasma however, the radioligand decreased rather quickly to 81.3% after an incubation time of 10 min in order to decrease further at a slower rate, resulting in 68.4 % after 2 hours. According to these experimental data radiochemical breakdown of ^{123}I-labelled annexin V could be attributed to the presence of proteases in plasma.

In vitro biological activity of [^{123}I]-annexin V

As shown in table 4 the percentage of [^{123}I]-annexin V binding to apoptotic PBL correlates for several samples with the degree of FITC annexin V binding which reflects on his turn the amount of apoptosis. However for the ceramide analogues, with exception of analogue E 41, there appears to be an overscore for the [^{123}I]-annexin V binding versus FITC annexin V binding.

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Apoptosis inducer	[^{123}I]-annexin V binding (%)	FITC annexin V binding (%)
control (untreated cells)	6.8	10.5
actinomycin D	39.9	69.0
γ-irradiation (5 Gy)	16.4	23.0
ceramide analogue E 41	9.5	15.0
ceramide analogue E 44	21.0	13.0
ceramide analogue F 9	12.2	9.0
ceramide analogue F17	13.6	12.0
ceramide analogue F21	39.4	15.0
control* (untreated cells)	36.0	40.0
cycloheximide*	47.6	60.0

Table 4. [^{123}I]-Annexin V binding versus FITC Annexin V binding 24 h after induction of apoptosis in PBL. *Binding percentages determined 48 h after apoptosis induction in PBL.

Concerning the apoptosis scores for actinomycin D (Figure 4), γ-irradiation (5 Gy), cycloheximide and the respective control samples, a better correlation (i.e. [^{123}I]-Annexin V binding versus FITC Annexin V binding was achieved when no washing step was introduced after incubation of the cell samples with [^{123}I]-Annexin V (Table 5).

Apoptosis inducer	[^{123}I]-Annexin V binding (%)	FITC Annexin V binding (%)
control (untreated cells)	8.3	10.5
actinomycin D	50.1	69.0
γ-irradiation (5 Gy)	21.4	23.0
control* (untreated cells)	40.5	40.0
cycloheximide*	49.8	60.0

Table 5. [^{123}I]-Annexin V binding versus FITC Annexin V binding 24 h after induction of apoptosis in PBL. *Binding percentages determined 48 h after apoptosis induction in PBL.

As a second control, several cell samples which underwent induced apoptosis were incubated with [^{123}I]-annexin V in the absence of [Ca^{2+}] in a similar way as described above. As the annexin V binding is known to be [Ca^{2+}] dependent, no significant binding appeared as expected (results not shown).

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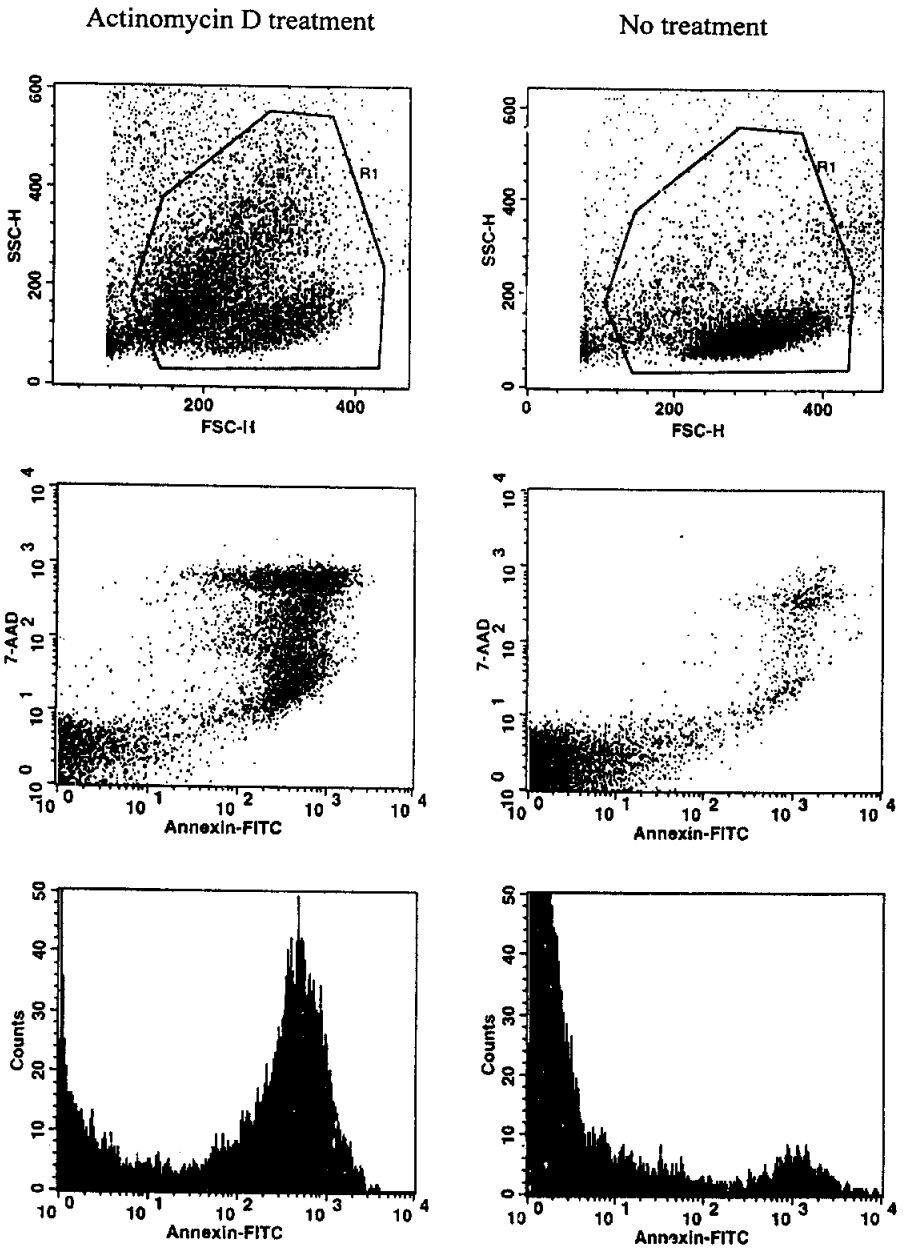

Figure 4. Scatterplots and annexin V histograms are shown for cells that were incubated with actinomycin D (left column) during 24 h in vitro and for cells that were incubated during 24 h also without any treatment. (FSC is forward scatter signal and SSC is side scatter signal).

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CONCLUSION

This paper shows that radioiodination of human recombinant annexin V using electrophilic substitution by means of Iodo-Beads can be quite successful in terms of labelling efficiency. Reaction parameters were optimized resulting in radiochemical yields up to 88 % as confirmed by TCA-precipitation and HPLC. After HPLC purification, the radiolabelled protein was obtained in a radiochemical purity of more than 95 % while specific activities were in the range of 10 MBq / μg of protein. In vitro stability results revealed that [^{123}I]-annexin V stays stable for at least 8 hours in phosphate buffer at physiological pH while in plasma a more pronounced degradation is noticed. However, this should not affect the suitability of the tracer for in vivo use if a favourable pharmacokinetical behaviour towards target tissues (i.e. a rapid blood clearance and a target / blood ratio > 1) could still be established. Furthermore, initial in vitro binding experiments with apoptotic cells indicate that [^{123}I]-annexin V remains its biological activity in comparison with FITC labelled annexin V. In conclusion, ^{123}I-labelled annexin V can be produced in sufficient amounts and high specific activities in order to find application as a SPECT-ligand in nuclear medicine for visualisation of apoptotic cells.

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