Imaging Myocardial Ischemia and Reperfusion Injury via Cy5.5-Annexin V

Rong Tian · DongFeng Pan

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Abstract

Aim The aim of this article is to present the results of an imaging study of myocardial apoptosis induced by ischemia/reperfusion injury.

Methods Twenty nude mice were randomly divided into an experimental group (10 mice) and control group (10 mice). In the experimental group, myocardial apoptosis was induced by ligation of the left anterior descending coronary artery (LAD) for 30 min. This was followed by reperfusion for 90 min. In the control group, the heart was exposed for the same length of time as in the experimental group. Cy5.5-annexin V (25 µg) was injected into both sets of mice after the onset of reperfusion. At 90 min post-injection, the mice were imaged. The region of interest (ROI) was obtained, and the fluorescence intensity of the ROI was quantified. The animals were sacrificed, and myocardial apoptosis was assayed by TUNEL assay.

Results Fluorescence intensity in the ischemia/reperfusion hearts was significantly higher than that in the control group ($P < 0.05$). In the TUNEL assay, more apoptotic cells were observed in the experimental group than in the control group, correlating with imaging results.

Conclusion Fluorescence imaging of Cy5.5-annexin V in a mouse model of myocardial ischemia/reperfusion can be used in vivo as a noninvasive means of detecting ischemia/reperfusion-induced apoptotic cells in the heart.

Keywords Myocardial ischemia/reperfusion · Apoptosis · Cy5.5-annexin V · Optical image

Introduction

Cardiomyocyte loss by cell death is dangerous because it causes irreversible damage to adult hearts owing to their very limited regeneration capability [1, 2]. Timely intervention following the exposure of myocardium to cardiotoxic events is critical for managing myocardial disease [3]. A number of animal and human studies have demonstrated that, in addition to necrosis, apoptosis significantly contributes to myocyte loss following ischemia and reperfusion (I/R) of the heart [1, 2, 3–6]. To define the therapeutic window of cell-death-blocking strategies, detailed information on the time frame of cell death after I/R of the heart is needed. In order to allow the selection of the appropriate interventions, noninvasive tests are required that enable the detection of myocardial damage. One of the earliest events after the triggering of apoptotic cell death is the externalization of phosphatidylserine (PS) to the outer leaflet of the plasma membrane of the cell. Detection of PS exposure can be easily achieved by the phospholipid binding protein annexin V [7, 8].

A number of in vitro and in vivo studies have demonstrated that annexin V is a specific marker for early and late stages of programmed cell death [8–13]. Radioisotope-labeled annexin V is suitable for the detection of cell death induced by ischemia and reperfusion injury in the myocardium [14]. Noninvasive optical imaging in small animals is undergoing significant advances due to the development of more sophisticated optical signal-detection devices and fluorophores with greater tissue-penetrating properties [15]. Optical imaging offers higher sensitivity and temporal resolution than nuclear imaging at the same spatial resolution

R. Tian (✉)
Department of Nuclear Medicine, Sichuan University,
37 Guoxuexiang,
Chengdu, China 610041
e-mail: zeyiqin@126.com

D. Pan (✉)
Radiology Department, University of Virginia,
P.O. Box 800170, Charlottesville, VA 22908, USA
e-mail: rt0705@126.com

in small animals. Optical imaging combined with a minimum of other techniques such as bioluminescence imaging and fluorescence imaging in the near infrared (NIR) spectrum could offer a signal-to-background ratio for detecting specific molecular signals that equals or exceeds that which can be achieved with other molecular imaging modalities [16–17]. An optical imaging technique for apoptosis would not only avoid the radiation and the need for frequent radiotracer synthesis but also might enable the visualization of apoptosis intraoperatively [19].

In the present study, we investigated whether NIR-fluorophore-labeled annexin V could image apoptosis induced by acute myocardial ischemia and reperfusion.

Materials and Methods

Material

Annexin V was obtained from BD Biosciences Pharmingen (San Diego, CA, U.S.). Cy5.5-bisfunctional succinimidyl ester was obtained from Amersham Biosciences (Piscataway, NJ, U.S.).

Labeling Annexin V with Cy5.5

Annexin V [1 ml, 30 nmol, 1 mg/ml in phosphate buffered saline (PBS) buffer, pH 7.4] was added to a vial containing 150 nmol of Cy5.5-bisfunctional succinimidyl ester; 500 μ l of 0.1 M sodium carbonate/bicarbonate buffer (pH 9.3) was also added to the vial. The reaction pH was 8.5. The vials were vortexed and incubated at room temperature for 30 min. The reaction mixtures were then purified by an Econo-Pac 10 DG column (Bio-Rad, Hercules, CA, U.S.). The conjugated Cy5.5-annexin V was eluted with PBS (pH 7.4) and was collected as a bluish band in the first fraction. The concentration of annexin V ($C_{\text{annexin V}}$) was determined by a modified Lowry protein assay reagent (Pierce Biotechnology, Rockford, IL, U.S.), and the conjugated Cy5.5 was determined by the absorbance at 675 nm (A_{675}). Both measurements were performed using a Biomate 5 UV/VIS spectrophotometer (ThermoSpectronic, Rochester, NY, U.S.). The final dye/protein (D/P) ratios were calculated using the following formula: $D/P = (A_{675}/\epsilon_{\text{Cy5.5}})/C_{\text{annexin V}}$, where the molar coefficient for Cy5.5, $\epsilon_{\text{Cy5.5}}$, was $215,410 \text{ mol}^{-1} \text{ L}^{-1} \text{ cm}^{-1}$, as determined by the absorption measurement of Cy5.5 at 675 nm (A_{675}) as a function of its concentration from 1.1×10^{-7} to $2.2 \times 10^{-5} \text{ M}$.

Binding Affinity of Cy5.5-Annexin V to Phosphatidylserine Measured by Surface Plasmon Resonance Spectrometry

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.). Small unilamellar vesicles (SUVs)

were prepared by bath sonication of a dispersion of lipids in 140 mM PBS buffer (pH 7.4). SUVs contained (by molar fraction) 80 % 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), 19.9 % 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and 0.1 % 1-oleoyl-2-(12-biotinyl(aminododecanoil))-sn-glycero-3-phospho-L-serine (biotin-DOPS) where indicated. SUV binding was monitored by surface plasmon resonance (SPR) spectrometry using a Biacore 3000 (GE Healthcare, USA). A streptavidin (SA) chip (GE Healthcare, USA) was mounted in the instrument, and SUV surfaces were created by capturing the SUVs on the Biacore SA chip at a 5 μ L/min flow rate via biotin-streptavidin interactions. SUVs were loaded onto the surfaces until 2,000 response units were achieved. The surfaces were stable and did not decay greatly during the titration.

Experiments were performed at 25°C with a 50 μ L/min flow rate. Proteins were diluted with HEPES-P buffer (0.1 M HEPES, 0.15 M NaCl, 0.005 % P20 surfactant), containing 5 mM CaCl_2 . Each surface of the biosensor was then exposed to 50- μ L injections of protein solution (association phase) followed by 250 μ L of buffer (dissociated phase) via the inject command. Between injections, a 5- μ L pulse of 50 mM Tris and 1 M NaCl (pH 8.0) solution was used to regenerate the SUV surfaces. Biacore data were analyzed by a 1:1 model using Biacore evaluation 3.1 software.

Evaluation of Cy5.5-Annexin V in Vitro

A single-cell suspension of thymocytes was prepared by mechanical separation from the thymus of a 6-week-old female BALB/c mouse. Two petri dishes were prepared by adding 2×10^7 thymocytes cells and 15 ml of RPMI 1640 medium (GIBCO, Grand Island, NY, U.S.) with 10 % FBS and 1 % antibiotics (penicillin/streptomycin). Then, 150 μ l of 10^{-6} M dexamethasone in DMSO was added to the first dish to induce apoptosis, and the other dish was kept as a control. The cultures were incubated overnight at 37°C with 5 % CO_2 . The cells were harvested by centrifugation at 500 g for 8 min, followed by washing twice with PBS buffer. After washing, cells were stained using a mixture of propidium iodine and FITC-annexin V (BD Pharmingen, CA, U.S.). The cell pellets were resuspended in 1 ml of annexin V binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl_2). Four hundred nanograms of either FITC-annexin V or Cy5.5-annexin V was added into each of the resuspended cell cultures in an Eppendorf vial. In a parallel experiment, cells were incubated with Cy5.5-annexin V. The solutions were incubated at room temperature in the dark for 15 min. Cells were analyzed using FACS Calibur (Flow Cytometry, BD Biosciences, USA). The signal difference between nonapoptotic and apoptotic cells was evaluated by the quotient of the medians of the M1 region of nontreatment cells and the M2 region of treatment cells.

Animal Model

Twenty nude mice were randomly divided into an experimental group (10 mice) and a control group (10 mice). In the experimental group, the mice were anesthetized under ketamine/xylazine (100 mg/kg) and were intubated perorally with a stainless steel tube. The mice were mechanically ventilated with room air. After left thoracotomy and exposure of the heart, the left anterior descending coronary artery (LAD) was ligated with 6-0 polypropylene just proximal to its main branching point. The suture was tied over a 1-mm polyethylene (PE-10) catheter, which was left in place during the planned period of ischemia (30 min). Blood flow was then reestablished (reperfusion) by removal of the tube. In the control group, the mice were anesthetized and intubated perorally with a stainless steel tube. Left thoracotomy was performed, and the heart was exposed for the same amount of time as in the experimental group.

Animal Imaging

A 25- μ g dose of Cy5.5-annexin V was injected into each mouse in the experimental group from the carotid artery at the onset of reperfusion. For the control group, the same dose of Cy5.5-annexin V was injected after left thoracotomy had been performed and the heart had been exposed for 30 min. At 30, 60, 90, and 120 min post-injection, the mice were imaged lying on their backs using the IVIS small-animal fluorescence-imaging system (Xenogen, Alameda, CA, U.S.) equipped with 150-W tungsten lamps and a cooled CCD camera. A set of bandpass filters customized for Cy5.5 with an excitation of 620–670 nm and emission wavelength of 695–770 nm was used. A mixture of 1 % isoflurane in oxygen was used to anesthetize the mice during imaging. Each mouse was imaged for 20 s. The region of interest (ROI) of the heart and the muscle close to the heart was obtained by manual drawing. The fluorescent intensity of the heart (H) and the muscle (M) were quantified with Living Image[®] software (Xenogen), and the H/M ratio was calculated for each mouse.

The animals were then sacrificed and the hearts were taken out and re-imaged *ex vivo*. This extensive series of images was obtained to confirm the source of the fluorescence signal detected by *in vivo* imaging.

Histology

After imaging, the mice were killed using an overdose of ketamine, and the hearts were removed, fixed in Bouin's fixative solution for 8 h, and embedded in paraffin. For the detection of apoptotic nuclei, the myocardium were sectioned at 3 μ m and stained using a terminal deoxynucleotidyl transferase-mediated UTP end labeling (TUNEL)

method according to standard procedures using a commercially available kit (R&D Systems, Minneapolis, MN, U.S.). The number of TUNEL-positive cells in each of the two groups was counted on five randomly selected 100 $\times$ fields for each section under a light microscope (Carl Zeiss, USA). Two nonconsecutive sections were analyzed per tumor sample.

Statistical Analysis

The unpaired Student's *t*-test was performed to evaluate the significance of differences in H/M values between the two groups. Correlations between the fluorescence uptakes and the number of TUNEL-positive cells were analyzed. All values are shown as the mean $\pm$ SD. A two-tailed value of $P<0.05$ was considered significant.

Results

Binding Affinity of Cy5.5-Annexin V to Membrane-bound PS as measured by SPR

The K_d values of unlabeled annexin V and Cy5.5-annexin V were 1.54×10^{-9} and 2.51×10^{-9} M, respectively, indicating that after being labeled with Cy5.5, the binding affinity of annexin V to PS did not change significantly when the final D/P ratio was around 2.

Evaluation of Cy5.5-Annexin V in Vitro

The results indicated dexamethasone treated and untreated cells showed two distinct populations of annexin V-stained cells, with the proportion of apoptotic cells increasing with dexamethasone treatment (Fig. 1). As expected, dexamethasone treatment induced apoptosis and increased the proportion of highly stained cells. Apoptotic cells had a near-infrared fluorescence (NIRF) that was higher than live cells. Additional double label flow cytometry experiments using FITC-annexin V and Cy5.5-annexin V indicated that both annexins reacted with the same cell population present in the cell culture.

TUNEL Staining

TUNEL stain images are shown in Fig. 2. The results showed that the number of TUNEL-positive cells in the experimental group was significantly higher than in the control group, 570 ± 76 vs. 215 ± 77 ($P<0.005$), respectively. This indicates that the histological sections obtained from the experimental group had a higher incidence of apoptotic cells than those obtained from the control group.

Fig. 1 a–d Histograms of thymocytes differentiated by FACS. *Top row* Cells without dexamethasone treatment. *Bottom row* Cells after apoptosis induction. **a** and **c** Cy5.5-annexin V; **b** and **d** FITC-annexin V. *M1* Viable cells, *M2* nonviable cells

Noninvasive Imaging of Myocardial Apoptosis

The images for different ischemia/reperfusion times (I/R) (30/30, 30/60, 30/90, and 30/120 min) were obtained. The results showed that the fluorescence was most intense in the 30/90 group. The results also indicated that myocardial cells in the experimental group had a greater uptake of fluorescence than those in the control group (Figs. 3 and 4).

Correlations between the fluorescence uptakes and the number of TUNEL-positive cells were analyzed. The TUNEL-positive cells correlated well with the fluorescence uptakes of myocardial cells ($r=0.7925$, $p<0.001$) (Fig. 5).

In all animals examined, ex vivo imaging of the hearts demonstrated that fluorescence intensity in hearts after IR was significantly increased over that in sham operated hearts (Figs. 3, 4).

Fig. 2 TUNEL staining (10 $\times$) of mouse heart specimens in ischemia/reperfusion (**a**) and in non-ischemia/reperfusion (**b**). Cells stained brown were considered TUNEL-positive. Specimens from **a** showed more positive cells than those from **b**

Fig. 3 **a–f** Images for different I/R durations. **a** Background (without injection of Cy5.5-annexin V), **b** 30/30 min, **c** 30/60 min, **d** 30/90 min, **e** 30/120 min, **f** heart removed (*L* control mouse, *R* experimental mouse). Fluorescence was most intense at 90 min post-injection. The myocardial cells in the experimental group showed more uptake of fluorescence than those in the control group

Discussion

The present study demonstrates that near-infrared fluorescence (NIRF)-labeled annexin V provides an alternative method for the detection of cell death in situ in an I/R model of the heart in mice. In our experiments, we found that there were no significant differences in the K_d values for affinity to PS between Cy5.5-annexin V and unlabeled annexin V, indicating that the addition of the Cy5.5 label did not

significantly decrease the binding affinity of annexin V to PS. In vivo imaging indicated that the myocardial cells in I/R mice had a greater uptake of fluorescence than those of the control mice, indicating that the myocardium in I/R mice contained more apoptotic cells than the control mice. These results were in accordance with those found by TUNEL staining. The TUNEL stain indicated that the histological sections obtained from the experimental group had a higher incidence of apoptotic cells than those from the control

Fig. 4 Uptake of fluorescence in myocardial cells for different I/R durations. The myocardial cells in the experimental group showed more uptake of fluorescence than those in the control group

Fig. 5 Correlations between the fluorescence uptakes and the number of TUNEL-positive cells

group. TUNEL-positive cells correlated well with the fluorescence uptakes of myocardial cells. Thus, the present study provides a noninvasive *in vivo* method for detecting myocardial apoptosis. To our knowledge, this is the first report about the application of near infrared fluorescence-labeled annexin V imaging in a myocardial I/R model.

Ewald and colleagues reported that after 15 min of ischemia followed by 30 min of reperfusion, annexin V-positive cardiomyocytes could already be observed in the reperfusion area (AR). Extending the reperfusion time to 90 min resulted in a marked increase in annexin V-positive cardiomyocytes. Further extension of the ischemic period to 30 min increased the percentage of annexin V-positive cardiomyocytes in the AR to 20.2 % [20]. So, in this study, 30 min ischemia followed by different reperfusion times was used to set up I/R models. The images for the different ischemia/reperfusion times (I/R) (30/30, 30/60, 30/90, and 30/120 min) indicated that fluorescence intensity increased with the duration of reperfusion and reached its peak at an I/R of 30/90 min. This suggests that the number of apoptotic cells was the highest in the mice subjected to 30 min of ischemia and 90 min of reperfusion. This is consistent with the results found by Dumont et al. who saw that the percentage of annexin V-positive cardiomyocytes in the at-risk area peaked in mice subjected to 30 min of ischemia and 90 min of reperfusion (I/R 30/90 mice) [4].

Apoptosis imaging provides a noninvasive *in vivo* method for detecting myocardial ischemia/reperfusion injury. Several categories of molecular probes have been applied experimentally and clinically for imaging detection of myocardial cell death [21–24], but annexin V is the most extensively used probe for imaging apoptosis in diverse clinical circumstances [25]. Radioisotope-labeled annexin V has

been used to detect cell death induced by ischemia and reperfusion injury in the myocardium [21, 22, 26]. However, it has the risk of exposing patients to radiation. NIRF-annexin V had been used for imaging cell death induced by chemotherapy [27]. To our knowledge there is no report on detecting cell death induced by ischemia and reperfusion injury in the myocardium.

Because of the limited ability of fluorophores to penetrate tissue, Cy5.5-annexin V imaging cannot be used on deep tissue. Its applicability to patient management must be investigated. With the progress of more sophisticated optical signal detection devices and fluorophores with greater tissue-penetration properties, this technique might be applicable in human beings before long.

In conclusion, we have demonstrated that Cy5.5-annexin V can noninvasively image injury-induced cardiomyocyte membrane alterations using *in vivo* fluorescence optical imaging. Near-infrared fluorescence (NIRF) imaging for apoptosis would not only avoid the risks of radiation exposure and the need for frequent radiotracer synthesis but also provide better sensitivity than radioactive imaging. With the recent progress in the fields of optical signal detection devices and fluorophores with greater tissue-penetrating properties, NIRF imaging of tissues lying deep within small or even large animals is now feasible.

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Conflict of interest We declare that we have no conflicts of interest.

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References

1. Fliss H, Gattinger D. Apoptosis in ischemic and reperfused rat myocardium. *Circ Res*. 1996;79(5):949–56.
2. Gottlieb RA, Bursleson KO, Kloner RA, Babior BM, Engler RL. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J Clin Invest*. 1994;94(4):1621–8.
3. Buja LM. Myocardial ischemia and reperfusion injury. *Cardiovasc Pathol*. 14(4):170–175.
4. Dumont EAWJ, Hofstra L, van Heerde WL, van den Eijnde S, Doevendans PAF, DeMuinck E, et al. Cardiomyocyte death induced by myocardial ischemia and reperfusion: measurement with recombinant human annexin-V in a mouse model. *Circulation*. 2000;102:1564–8.
5. Freude B, Masters TN, Robicsek F, Fokin A, Kostin S, Zimmermann R, et al. Apoptosis is initiated by myocardial ischemia and executed during reperfusion. *J Mol Cell Cardiol*. 2000;32(2):197–208.
6. Elsasser A, Suzuki K, Lorenz-Meyer S, Bode C, Schaper J. The role of apoptosis in myocardial ischemia: a critical appraisal. *Basic Res Cardiol*. 2001;96(3):219–26.
7. Blankenberg FG, Katsikis PD, Tait JF, Davis RE, Naumovski L, Ohtsuki K, et al. In vivo detection and imaging of phosphatidylserine expression during programmed cell. *Proc Natl Acad Sci USA*. 1998;95:6349–54.
8. Ohtsuki K, Akashi K, Aoka Y, Blankenberg FG, Kapiwoda S, Tait JF, et al. Technetium-99m HYNIC-annexin V: a potential radiopharmaceutical for the in-vivo detection of apoptosis. *Eur J Nucl Med*. 1999;26(10):1251–8.
9. Blankenberg FG, Katsikis PD, Tait JF, Davis RE, Naumovski L, Ohtsuki K, et al. Imaging of apoptosis (programmed cell death) with 99mTc annexin V. *J Nucl Med*. 1999;40(1):184–91.
10. Blankenberg F, Ohtsuki K, Strauss HW. Dying a thousand deaths. Radionuclide imaging of apoptosis. *Q J Nucl Med*. 1999;43(2):170–6.
11. Blankenberg FG, Tait J, Ohtsuki K, Strauss HW. Apoptosis: the importance of nuclear medicine. *Nucl Med Commun*. 2000;21(3):241–50.
12. Yang DJ, Azhdarinia A, Wu P, Yu DF, Tansey W, Kalimi SK, et al. In vivo and in vitro measurement of apoptosis in breast cancer cells using 99mTc-EC-annexin V. *Cancer Biother Radiopharm*. 2001;16(1):73–83.
13. Boersma HH, Kietselaer BL, Stolk LM, Bennaghmouch A, Hofstra L, Narula J, et al. Past, present, and future of annexin A5: from protein discovery to clinical applications. *J Nucl Med*. 2005;46(12):2035–50.
14. Blankenberg FG, Strauss HW. Noninvasive strategies to image cardiovascular apoptosis. *Cardiol Clin*. 2001;19(1):165–72, x.
15. Lin Y, Weissleder R, Tung CH. Novel near-infrared cyanine fluorochromes: synthesis, properties, and bioconjugation. *Bioconjug Chem*. 2002;13(3):605–10.
16. Luker GD, Luker KE. Optical imaging: current applications and future directions. *J Nucl Med*. 2008;49(1):1–4.
17. Napp J, Mathejczyk JE, Alves F. Optical imaging in vivo with a focus on paediatric disease: technical progress, current preclinical and clinical applications and future perspectives. *Pediatr Radiol*. 2011;41(2):161–75.
18. Hadjipanayis CG, Jiang H, Roberts DW, Yang L. Current and future clinical applications for optical imaging of cancer: from intraoperative surgical guidance to cancer screening. *Semin Oncol*. 2011;38(1):109–18.
19. Massoud TF, Gambhir SS. Molecular imaging in living subjects: seeing fundamental biological processes in a new light. *Genes Dev*. 2003;17:545–80.
20. Taki J, Higuchi T, Kawashima A, Tait JF, Kinuya S, Muramori A, et al. Detection of cardiomyocyte death in a rat model of ischemia and reperfusion using 99mTc-labeled annexin V. *J Nucl Med*. 2004;45(9):1536–41.
21. Dumont EA, Hofstra L, van Heerde WL, van den Eijnde S, Doevendans PA, DeMuinck E, et al. Cardiomyocyte death induced by myocardial ischemia and reperfusion: measurement with recombinant human annexin-V in a mouse model. *Circulation*. 2000;102(13):1564–8.
22. Sarda-Mantel L, Hervatin F, Michel JB, Louedec L, Martet G, Rouzet F, et al. Myocardial uptake of 99mTc-annexin-V and 111In-antimyosin-antibodies after ischemia-reperfusion in rats. *Eur J Nucl Med Mol Imaging*. 2008;35(1):158–65.
23. Wolters SL, Corsten MF, Reutelingsperger CP, Narula J, Hofstra L. Cardiovascular molecular imaging of apoptosis. *Eur J Nucl Med Mol Imaging*. 2007;34 Suppl 1:S86–98.
24. Bohm I, Heverhagen JT, Behe M, Greschus S, Willinek W, Lohmaier S, et al. Molecular imaging of apoptosis in cardiovascular diseases. *RoFo*. 2007;179(8):780–9.
25. Blankenberg FG. In vivo detection of apoptosis. *J Nucl Med*. 2008;49 Suppl 2:81S–95S.
26. Hofstra L, Leim IH, Dumont EA, Boersma HH, van Heerde WL, Doevendans PA, et al. Visualisation of cell death in vivo in patients with acute myocardial infarction. *Lancet*. 2000;356:209–12.
27. Schellenberger EA, Bogdanov Jr A, Petrovsky A, Ntziachristos V, Weissleder R, Josephson L. Optical imaging of apoptosis as a biomarker of tumor response to chemotherapy. *Neoplasia*. 2003;5(3):187–92.