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In vivo imaging of myocardial cell death using a peptide probe and assessment of long-term heart function

Bodhraj Acharya [a], Kai Wang [b], In-San Kim [a], WoongChol Kang [c], Chanil Moon [d,*], Byung-Heon Lee [a,**]

[a] Department of Biochemistry and Cell Biology, School of Medicine, Cell & Matrix Research Institute, Kyungpook National University, Daegu, Republic of Korea
[b] Department of Plastic Surgery, Henan Provincial People's Hospital, Zhengzhou, Henan, China
[c] Department of Cardiology, Gil Hospital, Gachon University, Incheon, Republic of Korea
[d] Department of Cardiology, School of Medicine, Hanyang University, Seoul, Republic of Korea

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ABSTRACT

During acute myocardial infarction (AMI), both apoptosis and necrosis of myocardial cells could occur and lead to left ventricular (LV) functional decline. Here we determined whether *in vivo* imaging signals of myocardial cell death by ApoPep-1 (CQRPPR), a peptide probe that binds to apoptotic and necrotic cells through histone H1, at an early stage after AMI showed correlation with the long-term heart function. AMI was induced using a rat model of ischemia and reperfusion (I/R) injury. Fluorescence-labeled ApoPep-1 was administered by intravenous injection into rats 2 h after reperfusion. *Ex vivo* imaging of hearts isolated 2 h after peptide injection showed higher levels of near-infrared fluorescence (NIRF) signals at hearts of I/R rats than those of sham-operated rats. The fluorescent peptide was rapidly cleared from the blood and did not bind to red and white blood cells. Localization of fluorescent ApoPep-1 at the area of cell death was demonstrated by co-staining of myocardial tissue with TUNEL. The intensity of *in vivo* NIRF imaging signals by homing of ApoPep-1 to injured myocardium of I/R rats obtained 2 h after peptide injection (equivalent to 4 h after injury) showed strong and moderate correlation with the change in the LV ejection fractions ($r^2 = 0.82$) and the size of the fibrotic area ($r^2 = 0.64$), respectively, observed at four weeks after injury. These results suggest that ApoPep-1-mediated *in vivo* imaging signals of myocardial cell death, including both apoptosis and necrosis, at an early stage of AMI could be a potential biomarker for assessment of long-term outcome of heart function.

1. Introduction

Left ventricular (LV) remodeling is the major determinant for development of congestive heart failure after acute myocardial infarction (AMI), which is a common presentation of cardiovascular disease. The magnitude of LV remodeling is affected by several factors, including age, sex, time to reperfusion, infarct size, and baseline LV volume indices. In AMI, the heart cells begin to die and collagen deposition ensues in its place [1]. Both apoptosis (dying) and necrosis (irreversibly dead) of myocardial cells could occur and lead to LV functional decline during AMI. Myocardial cell death also plays an important role in rejection after cardiac transplantation. Some studies have reported that cells are primarily apoptotic and thereafter become necrotic during AMI, while others have reported simultaneous appearance of necrosis and apoptosis [2–5]. Early reperfusion therapy for AMI is of great importance in order to reduce cell death and infarct expansion and for improvement of LV remodeling [6].

Recognition of reperfusion, the extent of cell death, and infarct size by non-invasive *in vivo* imaging would be useful in deciding on long-term treatment strategies and for prediction of LV remodeling and the prognosis in patients with AMI. Microvascular perfusion has been investigated by imaging techniques, such as intracoronary myocardial contrast echocardiography and magnetic resonance (MR) imaging [7–9]. Imaging of apoptosis has been performed using probes that recognize biomarkers exposed on the cell membrane or caspase enzymes activated in the cytoplasm during apoptosis [10–12]. A typical example is annexin V, which binds to the phosphatidylserine that is exposed on the surface of apoptotic cells [13]. Imaging of necrosis has been performed using probes, such as anti-myosin antibody, and has been based on the exchange of large molecules across the damaged cell membrane [12]. Infarct size has been assessed by late gadolinium enhancement at infarct tissue by MR imaging at an early stage in patients with AMI, which showed correlation with heart function and prognosis of patients [14,15].

ApoPep-1, a phage display-identified peptide with the sequence of CQRPPR, recognizes apoptotic cells by binding to histone H1 exposed on the surface of apoptotic cells [16]. In addition, it recognizes necrotic

* Correspondence to: C. Moon, Department of Cardiology, School of Medicine, Hanyang University, 17 Haengdang-dong, Seongdong-gu, Seoul 133-792, Republic of Korea.
** Correspondence to: B.H. Lee, Department of Biochemistry and Cell Biology, School of Medicine, Kyungpook National University, 101 Dongin-Dong, Jung-Gu, Daegu 700-421, Republic of Korea. Tel.: +82 53 420 4824.
E-mail addresses: cmoon351@gmail.com (C. Moon), leebh@knu.ac.kr (B.-H. Lee).

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cells by binding to histone H1 located at the nucleus, probably by entering necrotic cells across the damaged cell membrane [16]. Histone H1, a linker histone, is one of the most abundant proteins in the nucleus. Of interest, it is trans-located to the cytoplasm and becomes exposed on the cell membrane during apoptosis, thereby serving as a unique specific biomarker for cell death [17,18]. ApoPep-1 has been successfully used for imaging apoptotic cell death of tumor cells in response to cancer chemotherapy [16] and of neuronal cells in a Parkinson's disease model [19]. In addition, ApoPep-1 has been used as a targeting probe for apoptosis-targeted delivery of nanocarriers and T cells to tumor [20,21]. Potential advantages of small peptides as an imaging probe would be more efficient penetration into tissues and easier conjugation with imaging agents compared to proteins and antibodies.

In this study, we examined whether ApoPep-1 is a useful imaging probe for assessment of myocardial cell death by detecting both apoptosis and necrosis as a whole during AMI in which both apoptosis and necrosis are present together. We further examined whether ApoPep-1-mediated imaging signals of myocardial cell death at an early stage after AMI could be a potential biomarker for use in prediction of the long-term outcome of heart function.

2. Methods

2.1. Peptide synthesis and fluorescence labeling

ApoPep-1 (CQRPPR; molecular weight, 756 Da) and control peptide (NSSSVDK; molecular weight, 735 Da) were synthesized by Peptron Inc. (Daegeon, Korea). Fluorescein isothiocyanate (FITC) dye (molecular weight, 332 Da) was conjugated at the amino terminal of the peptide during synthesis. Flamma774 near-infrared fluorescence (NIRF) dye (molecular weight, 1060 Da) was conjugated at the amino terminal of the peptide by the manufacturer (Bioacts, Incheon, Korea). The excitation/emission wavelengths for FITC and Flamma774 were 490/520 nm and 778/805 nm, respectively.

2.2. Ischemia and reperfusion (I/R) injury and experimental scheme

Myocardial infarction was induced in ten rats by ligation and reperfusion of the left coronary artery (LCA). The ischemia and reperfusion (I/R) injury model is a widely used method, which closely resembles the pathological condition of AMI in humans [22,23]. Eight week-old male Sprague–Dawley rats (body weights 265 ± 11 g) were randomly divided into I/R or sham groups. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg) and were intubated. Thoracotomy was performed for opening of the chest and the heart was exposed. The paraconal interventricular branch of the LCA, corresponding to the left anterior descending coronary artery in humans, was ligated with 5-0 Ethicon-coated polyglactin suture. After 30 min of ligation, the artery was reperfused for 2 h. For the sham operation, rats underwent the same procedure with I/R rats except the ligation. All animal experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Kyungpook National University.

The experimental scheme is shown in Fig. 1. I/R rats were subdivided into three experimental groups: *ex vivo* imaging, microscopic analysis, and *in vivo* imaging. For *ex vivo* imaging, NIRF dye-labeled ApoPep-1 (200 nmol or 362 μg per rat) was administered into rats through the tail vein and circulated for 2 h. Hearts were isolated for NIRF imaging and 2,3,5-triphenyltetrazolium chloride (TTC) staining. For microscopic analysis, FITC-labeled ApoPep-1 (200 nmol or 218 μg per rat) was administered into rats through the tail vein and circulated for 2 h. Hearts were isolated and stained with transferase-mediated UTP end labeling (TUNEL) and anti-histone H1 antibody and then observed under a microscope. For *in vivo* imaging, Flamma774 NIRF dye-labeled ApoPep-1 was administered into rats through the tail vein. *In vivo* NIRF images were taken 2 h and 6 h after peptide injection. Echocardiography

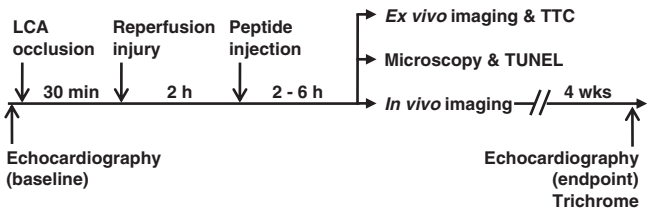

Fig. 1. Experimental scheme. Myocardial infarction was induced by 30 min of LCA ligation and 2 h of reperfusion. NIRF or FITC-conjugated ApoPep-1 was injected into the tail vein of rats and circulated for 2–6 h. Rats were divided into three experimental groups: *ex vivo* imaging; microscopic analysis; and *in vivo* imaging.

was performed before I/R injury (baseline) and at 4 weeks after injury (endpoint). After echocardiography, hearts were isolated and myocardial tissues were stained with Masson's trichrome.

2.3. Ex vivo and in vivo NIRF imaging of myocardial cell death

NIRF dye-labeled ApoPep-1 was injected into the tail vein of I/R and sham-operated rats after reperfusion. For *ex vivo* imaging, hearts were isolated 2 h after peptide injection and scanned for NIRF imaging signals using Optix exPlore™ (ART Inc., Montreal, Canada). *In vivo* NIRF imaging was performed 2 h and 6 h after peptide injection by scanning rats under anesthesia (enflurane in 80% N_2O, 20%O_2) using Optix exPlore™. Quantification of NIRF intensity in the region of interest (ROI) was performed by photon counting using Optix exPore Optiview™ software (version 1.0). ROI was defined by drawing a circle along NIRF signals at heart region. The same surface area of ROI was used for measuring NIRF signals of each rat.

2.4. Blood clearance kinetics and blood cell binding

After reperfusion, NIRF dye-labeled ApoPep-1 or control peptide was injected into the tail vein of I/R rats. Blood was collected at 5 min and 0.5, 1, 2, 4, and 6 h after peptide injection *via* tail vein in the presence of EDTA anticoagulant (2 mg/ml). A portion of the collected blood (20 μl) was blotted on the filter membrane. *Ex vivo* NIRF images of the filter membrane were taken and quantified using Optix exPlore™. Bovine serum albumin or histone H1 coated on a 96-well plate was incubated with FITC-labeled peptide at 4 °C for 1 h, and the fluorescence intensity was measured by a fluorometer. Red and white blood cells were isolated from the blood of a rat and incubated with FITC-ApoPep-1 at 4 °C and 37 °C for 1 h. The fluorescence of those cells was analyzed by a flow cytometer and fluorescence microscope, respectively.

2.5. Histological analysis of myocardial cell death and fibrosis

TTC staining was performed with frozen sections of heart tissues. TTC is reduced to red formazan in viable cells by a mitochondrial enzyme, while, in damaged cells, they become whitish in appearance due to loss of mitochondrial function. For detection of apoptotic cells, frozen sections of heart tissues were stained with the TUNEL reagent (Chemicon, Billerica, MA, USA) and observed under a fluorescence microscope. For co-localization analysis of TUNEL staining (red) and peptide binding (green), mean values of Mander's overlap coefficient were calculated by ImageJ software. Histone H1 staining was performed with paraffin-fixed heart tissues using a mouse anti-histone H1 antibody (Santa Cruz, CA, USA) at 4°C overnight. Fibrosis of myocardial tissue caused by infarct was assessed by Masson's trichrome staining [24]. The area of trichrome-stained, fibrotic tissue was measured using ImageJ software, which indicated the infarct size.