PET monitoring angiogenesis of infarcted myocardium after treatment with vascular endothelial growth factor and bone marrow mesenchymal stem cells

Mengting Cai¹ · Lei Ren¹ · Xiaqin Yin¹ · Zhide Guo² · Yesen Li² · Tingting He¹ · Yongxiang Tang¹ · Tingting Long¹ · Yutao Liu¹ · Gang Liu² · Xianzhong Zhang² · Shuo Hu¹

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Abstract Angiogenesis is a key factor for post-ischemic repair of the infarcted myocardium. This study aims to monitor angiogenesis of infarcted myocardium with a positron emission tomography (PET) imaging agent, ¹⁸F-alfatide II (¹⁸F-AIF-NOTA-E[PEG₄-c(RGDfK)]₂), targeting αvβ₃ integrin after treatment with vascular endothelial growth factor (VEGF) gene and/or bone marrow mesenchymal stem cells (BMSCs). Sprague–Dawley (SD) rats underwent left coronary artery ligation and were randomly divided into four groups: normal saline control, Ad-VEGF, BMSCs, and Ad-VEGF + BMSCs (n = 4/group). The induced myocardial infarction (MI) was confirmed by electrocardiogram (ECG) with ST-segment elevation, and ⁹⁹mTc-MIBI SPECT imaging showing defected myocardial perfusion. Alfatide II PET was performed to monitor angiogenesis at different time points after the therapy. The ratios of Alfatide II tracer uptake in the infarcted myocardium to normal myocardium in all four groups were analyzed. The PET results were validated by ex vivo tissue biodistribution, autoradiography, and immunofluorescence staining. At 1 week after therapy, elevated RGD peptide tracer uptake at the infarcted myocardium was observed in all four groups. The infarct to normal heart ratio of Alfatide II tracer for the three treatment groups was significantly higher than that of the control group (3.94 ± 0.20 for VEGF group, 3.77 ± 0.16 for BMSCs group and 4.86 ± 0.08 for the combination group vs. 3.01 ± 0.03 for the control group, P < 0.005, P < 0.005, P < 0.0001, respectively). The combination treatment group demonstrated higher contrast than the two single treatment groups. Similar results were also observed at 4 weeks after treatment. Autoradiography showed similar trend to that of PET results. Immunohistochemical staining showed expression of VEGF protein and the presence of adenovirus in the myocardium. The patterns of vascular density and integrin αvβ₃ expression were measured by CD31 and CD61 immunostaining analysis, and were consistent with the PET results. ¹⁸F-alfatide II PET could reflect angiogenesis of infarcted myocardium after VEGF gene and BMSCs therapy and further provide a non-invasive way of monitoring therapy response of myocardial infarction.

Keywords PET · Myocardial perfusion imaging · Myocardial infarction · Angiogenesis · VEGF · Mesenchymal stem cell · Integrin αvβ₃

Introduction

Myocardial infarction (MI) is one of the diseases with the highest morbidity and mortality worldwide. After MI, new blood vessels form, primarily either through angiogenesis or vasculogenesis, from the surrounding areas of the ischemic zone (van der Laan et al. 2009). Bone marrow mesenchymal stem cells (BMSCs) have been widely used in the treatment of MI, promoting myocardial repair (Huang et al. 2012). Homologous BMSCs transplantation to the heart muscle can differentiate into myocardial phenotypes, and promote angiogenesis through the secretion
of a variety of related factors such as vascular endothelial growth factor (VEGF) and angiogenin. VEGF gene therapy has specific effects on vascular endothelial cells to promote proliferation and angiogenesis. The combination therapy can promote endogenous VEGF secretion, resulting in increased angiogenesis and improved cardiac function within or near the infarcted myocardium (Pons et al. 2008; Halkos et al. 2008).

For the treatment of MI, it is vital to find a non-invasive method for real-time monitoring of the treatment efficacy. Positron emission tomography (PET) is one such method that has been widely used for its high sensitivity, relatively high resolution, and penetration capacity. In addition, it is a suitable modality for imaging in large animals and humans. Nuclear reporter gene imaging for monitoring of transplanted BMSCs in the body has been extensively studied. The distribution, survival, and prognosis of transplanted BMSCs in vivo can be non-invasively monitored by reporter gene imaging (Hu et al. 2011; Ylä-Herttuala et al. 2007; Nordlie et al. 2006). However, the method of monitoring the efficacy of treatment with BMSCs is limited. The angiogenesis of the infarcted myocardium after the treatment is rarely reported.

Integrins are a family of proteins that facilitate cellular adhesion and migration to extracellular matrix proteins. They are found in intercellular spaces and basement membranes, regulating cellular entry and withdrawal from the cell cycles (Niu and Chen 2011). During angiogenesis, integrins regulate the interaction between cells and extracellular matrix (ECM). The αvβ3 integrin plays a key role in endothelial cell survival and migration during angiogenesis. It is usually highly expressed on activated neovascular endothelial cells during angiogenesis after MI (Brooks et al. 1994), whereas the expression of αvβ3 integrin is low in mature endothelial cells and most normal organ systems. This feature makes it an ideal target for angiogenesis imaging.

18F-labeled RGD peptides through prosthetic groups, such as 18F-galacto-RGD, 18F-FPPRGD2, etc., have been advanced into clinic. However, the widespread use of these tracers may be limited due to the tedious and time-consuming labeling procedures with low labeling yield. The latest discovery and development of an 18F fluoride-aluminum complex to radiolabel peptides provides a unique method to much simplify the procedure (McBride et al. 2009, 2010). The whole labeling time was shortened from 2 to 3 h to 30 min without the need of HPLC purification. Considering the advantages of preparation and good imaging qualities, 18F-alfatide II (18F-AlF-NOTA-E[PEG4-c(RGDfK)]) is a promising tracer for PET imaging of integrin αvβ3 expression (Fig. 1).

In this study, we established a Sprague–Dawley (SD) rat MI model and subjected the animals to different treatments with adenovirus carrying VEGF gene, BMSCs, and the combination of adenovirus and BMSCs, respectively. Longitudinal PET imaging with 18F-alfatide II was performed to monitor the angiogenesis of MI. SPECT imaging with 99mTc-MIBI was used to confirm the success of the MI model and to observe the blood perfusion of the myocardium.

**Experimental section**

**General materials**

All reagents were of analytical grade and were obtained from commercial sources. 18F and 99mTc radionuclides were obtained from the First Affiliated Hospital of Xiamen University, and MIBI was obtained from Beijing Normal University. BMSCs were obtained from Cyagen Biosciences Inc.

**Preparation of 18F-alfatide II**

Radiolabeling of Alfatide II was performed with a similar procedure reported previously (Guo et al. 2014). The total synthesis time was about 30 min with radiochemical yield of 40–60 % and radiochemical purity >95 %. The specific activity was about 14.8–37 GBq/µmol at the end of synthesis based on the amount of peptide used and the amount of radioactivity trapped on the C-18 column.

**Adenoviral vector construction and production**

The GV314 vector, including enhanced green fluorescent protein (eGFP) (CMV-MCS-3FLAG-SV40-eGFP), was provided by Genechem Inc. (Shanghai, China). The vector was excised by BamHI/AgeI and connected with plasmid of vascular endothelial growth factor (VEGF). Then, the recombinant adenovirus (Ad-VEGF/eGFP) was transferred into 293T cells for amplification. The viral particles were collected and the titer was detected according to the manufacturer’s operating instructions (Genechem, Shanghai, China) after purification.

**BMSCs culture and characterization**

The rat BMSCs were purchased from Cyagen Biosciences Inc., and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) including 10 % fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. When the cells were proliferated to reach about 50 % confluence, differentiation incubation was started according to the instruction. To induce osteogenic differentiation of rBMSCs, the cells were cultured with osteogenesis culture medium
containing DMEM, 10% FBS, glutamic acid, 100 U/mL penicillin, 100 mg/mL streptomycin, 20 mM ascorbic acid, 1 M β-glycerophosphate, and 1 mM dexamethasone for 20 days. Then, the cells were observed under a microscope.

To investigate the potential of BMSCs to differentiate into adipose, the cells were cultured with adipogenic culture medium containing 5 mg/mL insulin, 250 mM 3-isobutyl-1-methylxanthine, 100 mM indomethacin, and 200 mL 1 mM dexamethasone for 16 days. Then, the cells were stained by oil red O and observed under a microscope.

Rat MI model and treatment

All animal experiments were conducted in accordance with the principles and procedures outlined in the NRC Guide for the Care and Use of Laboratory Animals and were performed under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the Central South University of China. 32 SD rats (adult male 250–300 g) were anesthetized with an intraperitoneal injection of 7% chloral hydrate. Before the rats were subjected to thoracotomy, they were intubated for mechanical ventilation by a small animal respirator (SAR-830/P Ventilator; CWE Inc., USA). A standard limb D1-D3 electrocardiogram (ECG) was connected to the limbs of rats with subcutaneous stainless steel electrodes. After the surgical site was shaved, a 2-cm incision was made in the fourth left intercostal space, and then the heart was exposed through a retractor inserting between the ribs and pericardial incision. Left anterior descending coronary arteries 2–3 mm from the tip of the left atrial appendage were permanently ligated with 6-0 polypropylene suture. ST-segment elevation myocardial infarction (STEMI) was recorded on an ECG monitor (RM6240B; Chengdu Instrument Factory), and visual regional cardiac cyanosis was used to confirm the occlusion and ischemia qualitatively.

Forty minutes after the coronary occlusion, the rats were randomly divided into four groups (n = 8/group), each group was immediately injected into three sites of the myocardium around the ischemic region (pale area): for the VEGF group, 60 μL adenovirus solution with 1 × 10^9 plaque forming units (PFU); for the BMSCs group, a total of 1 × 10^6 BMSCs in 60 μL of medium; for the VEGF + BMSCs combination group, 1 × 10^9 plaque forming units; and for the control group, 60 μL adenovirus vector without adenovirus solution. After 20 days, the rats were sacrificed, and their hearts were collected and used for further studies.

Fig. 1 Characterization of adenoviral vector and BMSCs. a Transfection of adenoviral vector Ad-VEGF/eGFP into 293T cells for 48 h causes strong eGFP fluorescence. b Left, calcium content is seen by alizarin red staining. Right, granular and drop lipid endocellular actuations are positively stained with oil red O (color figure online)
forming units (PFU) of adenovirus solution and $1 \times 10^6$ BMSCs in 60 $\mu$L of medium; and for the control group, 60 $\mu$L normal saline was injected. Recovery of animals was monitored after surgery.

**Micro-single photon emission computed tomography (SPECT) scan**

At 1 and 4 weeks after MI, $^{99m}$Tc-MIBI SPECT scans were performed at rest by using a Mediso nanoScan SPECT/CT. The rats were anesthetized with isoflurane (3 %), and approximately, 185 MBq (5 mCi) of $^{99m}$Tc-MIBI was administered via tail vein. Ten-minute static SPECT images were acquired at 1 h after injection of the radiotracer. Energy discrimination was provided by a 20 % window centered on 140.51 keV. SPECT data were obtained using a 4-headed gamma camera during a 360-degree rotation, and no attenuation or scatter correction was applied to this protocol. The SPECT program was applied to process short-axis tomograms to determine the location of the infarct area. The procedure was repeated two times.

**Small animal PET scan**

At 1 and 4 weeks after MI, the rats were anesthetized using 3 % isoflurane, temperature was maintained using a heating pad throughout the imaging procedures, and micro-PET scan was acquired for 15 min, starting at 1 h after an intravenous injection of approximately 37 MBq (1 mCi) of $^{18}$F-alfatide II. Acquisitions were performed using an Inveon microPET scanner (Siemens Preclinical Solutions). The data were acquired in three dimensions and stored in list-mode format and sorted into a three-dimensional sinogram, which was reconstructed using a two-dimensional ordered-subset expectation maximum (2D OSEM) algorithm, and no correction was applied for attenuation or scatter. The resulting matrix was $128 \times 128$ pixels, with 159 transverse slices.

In the PET imaging, regions of interest (ROIs) were drawn using vendor software (Inveon Research Workplace 4.1) on decay-corrected whole-body coronal images. The radioactivity concentrations in the heart were obtained from mean pixel values within the multiple ROI volume. Additionally, signal from the infarcted area was compared with the remote myocardium (at the septal level), which was taken as background signal, and expressed as the signal-to-background ratio.

The myocardium PET and SPECT images were fused with SPM software. To differentiate the signal of $^{18}$F-alfatide II in LV myocardial wall from the chest wall, ROI was drawn on the defect area of the MIBI myocardial perfusion in the fused image.

**Tissue biodistribution of $^{18}$F-Alfatide II**

At 1 week and 4 weeks after the myocardial infarction, rats were ($n = 4$/group) sacrificed, and the blood, myocardium, major organs, and tissues were collected, wet-weighed, and counted in an Automatic Gamma Counters (PerkinElmer Instruments, Inc.) The radioactivity of the tissue samples was converted to the percent injected dose per gram (%ID/g) after calibration.

**Autoradiography**

At the time of sacrifice, the rat hearts above were embedded and frozen in CRYO-OCT compound (Tissue-Tek) and cut transaxially at the location of the infarct. The apical part containing both infarct and non-affected contralateral myocardium was processed for sectioning for autoradiography. After an overnight exposure, the images were scanned in a Cyclone Plus Storage Phosphor System (PerkinElmer Instruments, Inc.).

The acquired photos were analyzed with Optiquant Acquisition and Analysis Software V5.0 (PerkinElmer Instruments, Inc.). ROI was manually defined for a focal myocardial tracer uptake region and a contra-lateral normal region on a mid-myocardial section. Radioactivity values of each ROI were measured in digital light units (DLUs)/per area ($\text{mm}^2$). DLU is an arbitrary linear unit that describes the intensity of photon emissions released during the scan. $^{18}$F-alfatide II uptake ratio was calculated by dividing the ROI value of the focal tracer uptake region by that of the contralateral normal area.

**Immunofluorescence staining**

At 1 week and 4 weeks after myocardial infarction, the rats were euthanized and their hearts were embedded and frozen in CRYO-OCT compound (Tissue-Tek). The embedded specimens were then sectioned (5 mm in thickness), fixed with ice-cold acetone for 10 min, and dried in the air for 30 min. Then, the frozen myocardial tissue slices were rinsed with phosphate-buffered saline for 2 min and blocked with 2 % bovine serum albumin for 30 min at room temperature. Resting endothelial cells were traced by CD31 antibody (BD Pharmingen™), and activated neovascular endothelial cells by CD61 antibody (BD Pharmingen™). The slices were then incubated with mouse anti-rat CD31 and CD61 antibody overnight at 4 °C and visualized using Cy3- conjugated mouse anti-rat secondary antibody. After three more washes with PBS, the slides were stained for nuclei with 4',6-diamidino-2-phenylindole (DAPI). Then, they were observed by an epifluorescence microscope. The fluorescence integrated density (IntDen) of CD31 was
quantified with the software of image-Pro Plus 6.0. And then, the average IntDen was worked out.

Hematoxylin and eosin (HE) and immunohistochemistry staining

At 1 week after myocardial infarction, the heart of each rat was excised, fixed in 10% formalin, embedded in paraffin, sectioned into 4 µm-thick slices, and placed on glass slides. Tissue samples were stained with both hematoxylin and eosin (HE) and VEGF (Santa Cruz Biotechnology, Inc.) antibody by use of standard techniques to monitor the expression of VEGF in myocardium.

Statistical analysis

Numerical data were expressed as mean ± standard deviation (SD). For comparison between 2 time points or groups, a Student’s t test was used. A P value of <0.05 was considered statistically significant.

Results

Adenoviral vector construction and production

To confirm successful transfer of adenoviral vector AdVEGF/eGFP into 293T cells, the cells were observed under fluorescence microscope at 48 h after transfection. The transfected cells could be seen with strong fluorescence (Fig. 1a).

Characterization of BMSCs

BMSCs were incubated in osteoblastogenic medium to examine the osteoblastogenic differentiation ability. During the observation period, the number of the cells kept increasing. And calcium content could be observed by alizarin red staining among cells after they were incubated for 20 days. At the same time, BMSCs were cultured with adipogenic culture medium to examine the adipogenic differentiation potential. After 16 days of adipogenic induction, cells were induced to form adipocytes. Many BMSCs contain granular and drop lipid intracellular actuations positively stained with oil red O (Fig. 1b).

Characterization of rat MI model

As shown in Fig. 2a, ECG confirmed the success of MI by ST segment elevation after ischemia. Seven days after myocardial infarction, obvious uptake defect in the anterior and lateral wall of left ventricle was observed with $^{99m}$Tc-MIBI SPECT.

$^{18}$F-Alfatide II PET imaging

To evaluate angiogenesis after myocardial infarction, PET imaging was performed at 1 week and 4 weeks after ischemia using $^{18}$F-Alfatide II as the imaging probe. Focally increased tracer uptake was seen in the anterior and lateral left ventricle wall and the chest wall. Increased $^{18}$F-alfatide II uptake was seen throughout the area of $^{99m}$Tc-MIBI defect. Representative myocardial transaxial PET images with $^{18}$F-Alfatide II are presented in Fig. 2b. On the contrary, the sham-operated rats had almost no $^{18}$F-Alfatide uptake in the heart region.

At 1 week after myocardial infarction, the infarct area/remote area uptake ratio of $^{18}$F-alfatide II in the control group was lower than that of the other three treatment groups (3.01 ± 0.03 vs. 3.94 ± 0.20 (VEGF group), P < 0.005; 3.77 ± 0.16 (BMSCs group), P < 0.005; and 4.86 ± 0.08 (combination group), P < 0.0001). Additionally, the uptake ratio of treatment group with intramyocardial co-injection of Ad-VEGF and BMSCs was higher than that with single injection of Ad-VEGF or BMSCs (P < 0.005). No obvious difference was found between the Ad-VEGF and BMSCs groups. The same trend was observed at 4 weeks after therapy. Compared with the control group (2.86 ± 0.13), the uptake ratio was significantly higher in the other treatment groups (3.44 ± 0.11 for Ad-VEGF group, P < 0.05; 3.51 ± 0.05 for BMSCs group, P < 0.05; and 4.48 ± 0.11 for combination group, P < 0.001) respectively (Fig. 2c).

Biodistribution

The biodistribution of $^{18}$F-alfatide II in MI rats is shown in Fig. 3. Data were acquired at 90 min after injection of the tracer. The entire heart was tested to further determine and compare $^{18}$F-alfatide II uptake. The hearts were collected at the same time points to provide a parallel reference for PET images and autoradiography results.

At 1 week after ischemia, whole heart uptake in three treatment groups (0.23 ± 0.02 %ID/g (VEGF group), 0.22 ± 0.003 %ID/g (BMSCs group), and 0.29 ± 0.01 %ID/g (combination group)) was higher than that of the control group (0.16 ± 0.01 %ID/g, P < 0.05, P < 0.05, and P < 0.005), respectively, and the tracer uptake in the combination treatment group was also higher than that in the two single treatment groups (P < 0.05) respectively, while the two single treatment groups showed no differences. After 4 weeks, $^{18}$F-alfatide II uptake of the injured hearts in the control group (0.13 ± 0.01 %ID/g) was lower than the three treatment groups (0.19 ± 0.01 %ID/g (VEGF group), 0.19 ± 0.01 %ID/g (BMSCs group), and 0.25 ± 0.02 %ID/g (combination group), P < 0.05,
However, no obvious differences were noted among the three treatment groups at this time point.

**Autoradiography**

In parallel to in vivo PET imaging, we also performed autoradiography to validate the quantitative results based on PET images. Typical autoradiographic images and averaged uptake ratios at 1 week and 4 weeks after MI are shown in Fig. 4a. At 1 week after MI, increased signal intensity was obvious in the infarcted and ischemic areas. The results confirmed the localized high level of 18F-alfatide II uptake as presented by non-invasive PET imaging. Quantification data demonstrated that the trend of uptake ratio of 18F-alfatide II corroborates with PET imaging results.

At 1 week after MI induction, the infarcted area/remote area uptake ratio in the control group (1.89 ± 0.13) was lower than that of the other three treatment groups (2.51 ± 0.07 (VEGF group), 2.48 ± 0.20 (BMSCs group), and 2.88 ± 0.06 (combination group), and the ratio of VEGF + BMSCs group was higher than that of VEGF and BMSCs single treatment groups ($P < 0.05$, $P < 0.001$). No significant difference between VEGF and BMSCs groups was found and the same situation appeared at 4 weeks after therapy.
CD31 and CD61 immunostaining

To further study the mechanism of increased $^{18}$F-alfa-tide II uptake post-MI, we performed CD31 and CD61 immunostaining to follow the changes of microvasculature and integrin β3 expression at different time points after ischemia. Results from CD31 and CD61 immunostaining are presented in Fig. 4b. Endothelial CD31 expression was...
observed in the normal as well as in the peri-infarct and infarcted areas. CD61 immunostaining was observed in the infarcted and peri-infarct zones, but not in the remote myocardium. At 1 week after surgery, the hearts in the treatment groups showed high level of CD31 and CD61 signals, and in the control group, low levels of CD31 and CD61 were found. Remarkably, stronger and more diffuse CD31 and CD61 signals were found in the hearts treated with VEGF + BMSCs than those with single VEGF or BMSCs treatment. At 4 weeks after myocardial infarction, the CD31 and CD61 signals were relatively lower than those at 1 week after ischemia.

The average IntDen of CD31 was 0.1136 ± 0.0048, 0.1322 ± 0.0057, 0.1229 ± 0.0036, 0.15479 ± 0.0011 in the control group, VEGF, BMSCs, and the combination group, respectively, at 1 week after the myocardial infarction, and that was 0.07816 ± 0.0014, 0.1298 ± 0.001, 0.1278 ± 0.0027, and 0.1318 ± 0.0007 in the control group and other three treatment groups, respectively, at 4 weeks after the myocardial infarction. The average IntDen of CD31 in the control group was obviously lower than that in VEGF group and BMSCs group, (P = 0.012, P = 0.019 respectively). And also significantly lower than that in the combination group, (P = 0.006). There was no statistical difference of IntDen of CD31 between VEGF group and BMSCs group. The results at 4 weeks were similar with those at 1 week after the myocardial infarction.

HE staining and Ad vector in myocardium

The results of HE staining in normal myocardium and infarcted myocardium are shown in Fig. 5a. To investigate whether the VEGF packaged in adenovirus was expressed in myocardium, we first performed VEGF immunohistochemical staining to follow the expression of VEGF after treatment. As shown in Fig. 5b, in the hearts from the adenovirus packaging VEGF and VEGF/BMSCs groups, positive VEGF staining was distributed extensively within myocardium, while in the ischemic myocardium treated by saline or BMSCs, no VEGF signal was detected. Then, the expression of eGFP in the frozen myocardial tissue slices was observed by a fluorescence microscope. As expected, no eGFP signal was found in the saline control and BMSC-treated animals, while strong and diffusive eGFP signal was seen in the other two groups (Fig. 5c).

Discussion

On account of its vasculoprotective and angiogenic properties, VEGF has been chosen as a therapeutic gene (Isner 1998). There is strong evidence that transplantation of therapeutic stem cells could enhance myocardial blood flow and improve cardiac function in ischemic heart disease (Segers and Lee 2008). VEGF gene therapy eight-year safety follow-up of CAD patients was also reported (Hedman et al. 2009).

In a porcine MI model, it has been found that VEGF expressing autologous cell therapy significantly increased wall thickness of the scar in the heart after myocardial ischemia (Hagikura et al. 2010). The treatment of Baculovirus-transduced, VEGF-expressing adipose-derived stem cell sheet prolonged VEGF expression, suppressed left ventricle (LV) remodeling, and preserved the LV geometry (Yeh et al. 2014). BMSC-based VEGF gene therapy strategies to achieve repair and regeneration of damaged tissues have yielded some positive outcomes. Moon et al. demonstrated that VEGF-BMSC transplantation could decrease the ratio of fibrotic area to LV area and infarcted to non-infarcted LV in ischemic myocardium as compared to BMSC transplantation. Therefore, BMSC-based VEGF gene therapy approach is capable of enhancing cardioprotective effect (Moon et al. 2014). However, gene therapy combined with stem cell therapy has been barely studied. In this study, co-treatment with Ad-VEGF and BMSCs can be valuable as it promotes more neointima formation than single treatment.

The expression of integrin α₃β₃ is low in most normal organ systems and mature endothelial cells, while it is highly expressed on activated neovascular endothelial cells during angiogenesis. It makes integrin α₃β₃ an ideal target for imaging to follow the myocardial infarction healing process. In the past few years, different cyclic RGD peptide-based tracers have been designed to image myocardial repair processes (Meoli et al. 2004; Higuchi et al. 2008; Gao et al. 2012; Sherif et al. 2012; Laitinen et al. 2013).

The preliminary clinical data revealed that RGD PET can reflect the extent of α₃β₃ expression within the infarcted area of patients with myocardial infarction and PCI (Makowski et al. 2008). At follow-up scans, tracer uptake was visualized in the infarcted region in the patients who still had symptoms, which was no longer noticeable in symptomless cases (Luo et al. 2014). The PET images showed that ¹⁸F-RGD signal corresponds to the regions of severely reduced ¹³N-ammonia flow signal (myocardial blood flow).

In this study, we applied for the first time a PET tracer ¹⁸F-alfatide II to visualize and quantify the temporal changes of integrin α₃β₃ expression upon different therapies, especially the co-treatment of Ad-VEGF and BMSCs, to evaluate the therapeutic effect. The imaging of angiogenesis after MI using a high-resolution micro-PET scanner resulted in strong contrast between infarcted and non-infarcted myocardium, and this study demonstrated that the uptake of a novel dimeric RGD peptide tracer ¹⁸F-Alfatide II was significantly higher in Ad-VEGF- and BMSCs-treated rats, than that in
PET monitoring angiogenesis of infarcted myocardium after treatment with vascular…

the control group. In particular, highest uptake of Alfatide II was found in the ischemic myocardium with Ad-VEGF and BMSCs co-treatment, compared with single treatment, reflecting enhanced cardioprotective effects of co-treatment over single treatment, which was also confirmed by immunohistochemical staining results.

It is of note that there are discrepancies between the biodistribution results at 4 weeks and the PET and autoradiography quantification. Direct tissue sampling studies showed higher whole heart uptake at 4-week time point of the treatment groups over the control group, but no difference among the three treatment groups. PET and autoradiography, on the other hand, showed higher infarct/normal myocardium ratio in the co-treatment group than that in the single-treatment groups. For biodistribution study, we used gamma counter to measure the radioactivity of the entire heart. While for PET and autoradiography, we were able to manually draw the infarcted myocardial tissue and the remote healthy myocardium, and thus we believe that the in vivo PET and ex vivo autoradiography are better measures of myocardium angiogenesis than the gross biodistribution studies by measuring the activity accumulation in the whole heart.

$^{99m}$Tc-MIBI SPECT imaging was used to confirm the presence of infarct and validate PET images and autoradiography results. Normal myocardial tissue showed high $^{99m}$Tc-MIBI uptake. Infarcted myocardium showed obvious $^{99m}$Tc-MIBI uptake defect in the anterior and lateral wall of left ventricle, which matched the focal RGD peptide tracer uptake region.

In conclusion, we demonstrated successful in vivo assessment of the angiogenesis in myocardial repair after MI and compared the beneficial effect of gene therapy, stem cell therapy, and also the combination therapy. Our results showed that combined Ad-VEGF and BMSCs treatment led to more microvessel density and higher integrin expression than either Ad-VEGF or BMSCs treatment.

Fig. 5  a HE staining of normal areas and infarct zone at 1 week after surgery (inset ×100 magnification). b Immunohistochemical staining of VEGF expression. Strong VEGF signals were found in VEGF and BMSCs/VEGF groups, but not in NS and BMSCs groups. c Immunofluorescence signals of eGFP in representative myocardial infarction sections. Strong and diffusive eGFP signals in VEGF and BMSCs/VEGF groups, and no signals in NS and BMSCs groups at 1 week after surgery.
alone. The therapeutic effect was successfully monitored by longitudinal Alfatide II/PET and $^{99m}$Tc-MIBI/SPECT. The findings of this study may warrant the development of future theranostics of myocardial infarction in patients.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare no competing financial interest.

**Statement on the welfare of animals** All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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