Evaluation of ^{68}Ga-Labeled Peptide Tracer for Detection of Gelatinase Expression after Myocardial Infarction in Rat

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Keywords:	Matrix metalloproteinase, Myocardial infarction, Positron emission tomography

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Abstract

Background. Matrix metalloproteinases 2 and 9 (MMP-2/9) play a role in extracellular matrix remodeling after an ischemic myocardial injury. We evaluated ^{68}Ga-DOTA-peptide targeting MMP-2/9 for the detection of gelatinase expression after myocardial infarction (MI) in rat.

Methods. Rats were injected with 43 ± 7.7 MBq of ^{68}Ga-DOTA-peptide targeting MMP-2/9 at 7 days (n=7) or 4 weeks (n=8) after permanent coronary ligation or sham operation (n=5 at both time-points) followed by positron emission tomography (PET). The left ventricle was cut in frozen sections for autoradiography and immunohistochemistry 30 minutes after tracer injection.

Results. Immunohistochemical staining showed MMP-2 and MMP-9 expressing cells, CD31 positive endothelial cells, and CD68 positive macrophages in the infarcted myocardium. Autoradiography showed increased tracer uptake in the infarcted area both at 7 days and 4 weeks after MI (MI-to-remote area ratio 2.5±0.46 and 3.1±1.0, respectively). Tracer uptake in damaged tissue correlated with the amount of CD68 positive macrophages at 7 days after MI, and CD31 positive endothelial cells at 7 days and 4 weeks after MI. The tracer was rapidly metabolized, radioactivity in the blood exceeded that of the myocardium and tracer accumulation in the heart was not detectable by *in vivo* PET.

Conclusions. ^{68}Ga-DOTA-peptide targeting MMP-2/9 accumulates in the damaged rat myocardium after an ischemic injury, but tracer instability and slow clearance *in vivo* makes it unsuitable for further evaluation.

Key Words: matrix metalloproteinase, myocardial infarction, positron emission tomography

Abbreviations

DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
ECM	Extracellular matrix
LCA	Left coronary artery
LV	Left ventricle
MI	Myocardial infarction
MMP	Matrix metalloproteinase
PET/CT	Positron emission tomography/computed tomography
PSL	Photostimulated luminescence
ROI	Region of interest
SUV	Standardized uptake value

INTRODUCTION

Matrix metalloproteinases (MMPs) are proteolytic enzymes that play a central role in the degradation of extracellular matrix (ECM) proteins in myocardial infarction (MI), healing process, and subsequent left ventricle (LV) remodeling[1,2,3]. Different MMP types have diverse spatial and temporal activation patterns in the heart after MI[3,4,5]. In particular, the expression of gelatinases MMP-2 and MMP-9 is elevated after an ischemic myocardial injury[4,5,6] in association with the early injury responses, such as inflammation and neovascularization[1,7], as well as post-MI remodeling[8,9]. While MMP-9 is activated during the first days after an ischemic injury, MMP-2 activation occurs later and persists longer during the post-MI remodeling phase[4,10].

Molecular imaging facilitates *in vivo* evaluation of MMP activation within the myocardium after MI[5,6,10]. Chen and co-workers demonstrated the feasibility of detecting MMP activity after MI using a near-infrared fluorescent probe that is activated upon cleavage by MMP-2/9[10]. Thereafter, radiolabeled broad-spectrum MMP-inhibitor has been used to visualize MMP activation after MI with the single-photon emission computed tomography (SPECT)[5,6]. Given the differences in activation patterns and functions of different MMP types within the post-MI myocardium, methods to assess expression of specific MMPs would be warranted.

In this study we have used a previously developed MMP-2/9 targeting positron emission tomography (PET) imaging agent (Figure 1)[11]. The peptide was identified from phage display library and selected based on its ability to bind chemically activated MMP-9[11,12] and shown to inhibit both MMP-9 and MMP-2[12] . The ^{68}Ga-labeled peptide tracer provides good target-to-background ratio for PET imaging of tumor xenografts expressing MMP-9 in rats[11]. Purpose of this study was to evaluate the feasibility of ^{68}Ga-labeled 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid conjugated MMP-2/9 targeting tracer (^{68}Ga-DOTA-peptide) for studying the distribution and extent of gelatinase expression during MI healing process and post-MI LV remodeling. Immunohistochemistry was used to detect MMP-9 expressing cells in the

myocardium together with other biological processes associated with ECM remodeling following acute MI. Biodistribution and myocardial uptake of the MMP-2/9 targeting ^{68}Ga-DOTA-peptide were studied by *in vivo* PET/computed tomography (CT) as well as radioactivity measurements and autoradiography of tissue samples. We hypothesized that with the specific probe we could monitor myocardial injury responses involving MMP-2/9 during early healing and remodeling phases after MI.

MATERIALS AND METHODS

Animal Model and Study Design

MI was induced by permanent surgical ligation of the left coronary artery (LCA) according to previously described procedures[13]. Briefly 0.2 mg/kg of buprenorphine (Temgesic; Schering-Plough, Espoo, Finland) was administered intramuscularly prior to operation for analgesia, the rats were anesthetized with a combination of inhaled isoflurane (Vet Medic Animal Health, Parola, Finland) (induction only) and subcutaneous injection of 10 mg/kg of xylazine (Rompun; Orion Pharma, Espoo, Finland) and 90 mg/kg of ketamine (Ketaminol; Orion Pharma, Espoo, Finland), intubated, connected to a respirator, thoracotomy was performed and suture was placed in the proximal LCA. The sham operation consisted of the same procedures except coronary ligation. A total of 65 male Sprague-Dawley rats ageing 7±1 weeks and weighing 330±71 g were used. Mortality was approximately 25% after either coronary ligation or sham-operation and occurred during the first two days after surgery. Animals that did not develop MI (scar < 4% of the LV circumference) were excluded from the study.

The main study group for evaluation of myocardial uptake of ^{68}Ga-DOTA-peptide consisted of 7 rats studied at 7 days and 8 rats studied at 4 weeks after coronary ligation; and 5 rats studied at 1 week and 5 rats at 4 weeks after the sham-operation. 3 unoperated rats were also used as controls. Rats were intravenously (i.v.) injected with 43±7.7 MBq (6.9±3.7 nmol) of ^{68}Ga-DOTA-

peptide via tail vein and imaged by dynamic PET/CT for 20 minutes starting at the time of injection. Rats were killed 30 minutes post-injection by cervical dislocation and tissue samples were obtained for measurement of tracer uptake by gamma counter or autoradiography as well as for histology.

In order to assess the specificity of ^{68}Ga-DOTA-peptide accumulation in the heart 7 days after coronary ligation, 5 rats were i.v. injected with ≥1.4 μmol/kg (approximately ≥20 μmol/L of blood volume, IC_{50}=10 μmol/L) of specific MMP-2/9 inhibitor [H-Cys^{1}-Thr-Thr-His-Trp-Gly-Phe-Thr-Leu-Cys^{10}-OH (cyclic: 1→10)] (product number 444251, Merck KGaA, Darmstadt, Germany) 5 minutes prior to the administration of ^{68}Ga-DOTA-peptide. In addition, 3 rats were injected with ~500 fold amount (compared to administrated ^{68}Ga-DOTA-peptide), of unlabeled peptide.

Tracer biokinetics were evaluated for a longer period of time by *in vivo*, i.e. a 60-min dynamic PET/CT scan was performed for 3 rats at 4 weeks after coronary ligation, 1 sham-operated rat, and 3 healthy control rats. These rats were i.v. injected with 42±6.7 MBq (15±8.6 nmol) of ^{68}Ga-DOTA-peptide.

The study protocol was approved by the National Animal Experiment Board in Finland and the Regional State Administrative Agency for Southern Finland, and carried out in compliance with the relevant European Union directives.

Radiochemistry and *In Vivo* Stability

The DOTA-conjugated peptide (Figure 1) was purchased from Peptide Specialty Laboratories GmBH (Heidelberg, Germany) and ^{68}Ga labelled as previously described[11]. The *in vivo* stability of the ^{68}Ga-DOTA-peptide was studied in 2 rats at 4 weeks after coronary ligation until 60 minutes post injection of 49±3.8 MBq. Detailed description of labeling and analyses are described in the electronic supplementary material.

PET/CT Image Analysis

The rats were imaged using a small-animal PET/CT (Inveon Multimodality; Siemens Medical Solutions, Knoxville, TN, USA) under isoflurane anesthesia. The full width at half maximum resolution of the scanner for ^{68}Ga is 2.46 mm.[14] PET data was acquired for 20 or 60 min starting at the time of injection of ^{68}Ga-DOTA-peptide. Immediately after PET, 200-µL of intravascular iodinated contrast agent eXIA^{TM}160XL (Binitio Biomedical Inc, Ottawa, ON, Canada) was injected i.v. and high-resolution CT was acquired. Detailed protocols are described in the supplement. Images were analysed using Carimas v.2.6 software (Turku PET Centre, Turku, Finland). Alignment of PET and CT images was automatic and confirmed by anatomical landmarks. Data was normalized and corrected for injected radioactivity dose and radionuclide decay. Regions of interest (ROIs) were drawn according to high-resolution CT image in the infarcted myocardium (or corresponding location in the anterior wall in sham operated rats), remote myocardium in the septum, blood pool (inside the LV cavity), and skeletal muscle. Results were reported as mean radioactivity concentration (Bq/mL converted to standardized uptake values [SUV]) as a function of time after injection, i.e. as time-activity curves.

***Ex Vivo* Biodistribution**

A blood sample was obtained by cardiac puncture. Then, the LV (rinsed with saline and without atria or the right ventricle) and various other tissues were excised, weighed and measured for radioactivity using a gamma counter (Triathler 3", Hidex, Turku, Finland). Results were expressed as SUV.

Autoradiography

The LV was frozen in isopentane and sliced into serial 8- and 20-µm transverse cryosections from apex to base for analysis of ^{68}Ga-DOTA-peptide uptake by digital autoradiography as previously described[13]. Radioactivity accumulation was measured in 20-µm sections, which provided better count statistics. Autoradiographs were co-registered with images of the same tissue sections stained with hematoxylin and eosin (HE). Based on histology, ROIs were defined as the infarcted area (covering the whole infarcted region) and the remote, non-infarcted area in the posterior or inferior wall of the LV. For more details see electronic supplementary material.

Histology and Immunohistochemistry

Serial LV cryosections were hematoxylin and eosin (HE) stained for general histology. Masson's trichrome (Sigma-Aldrich, St. Louis, MO, USA) staining was used to distinguish fibrosis and collagen from cardiomyocytes. Macrophages, endothelial cells, MMP-2 and MMP-9 positive cells were detected by immunohistochemical staining using the following primary antibodies: mouse monoclonal anti-rat CD68 (dilution 1:10000, Product N:o MCA341GA, AbD Serotec, Munich, Germany), monoclonal anti-rat CD31 (dilution 1:10000, Product N:o MCA1334GA, AbD Serotec), polyclonal anti-MMP-2 (dilution 1:100, Product N:o ab19167, Merck, Darmstadt, Germany) and polyclonal anti-MMP-9 (dilution 1:1000, Product N:o ab38898, Abcam, Cambridge, UK). Double stainings with anti-MMP-9 (1:1000) and either CD68 (1:15000) or monoclonal antibody for α-smooth muscle actin (1:10000, Product N:o A5228, Sigma Aldrich) were performed in several sections to identify cell types expressing MMP-9.

Digital images of the stained sections were captured with 3D Histech Pannoramic 250 Flash digital slide scanner (3D Histech, Budapest, Hungary). Size of the MI was measured in Masson's trichrome stained sections with Image-J v. 1.46 software (National Institutes of Health, Bethesda, MD) as circumferential percentage of the infarct scar of the whole endocardial length of the LV. Furthermore, percentages of myocardium positive for CD68 or CD31 or collagen (blue color in

Masson's trichrome staining) were quantified within the infarcted and remote areas with the use of Image-J software and specific color thresholds.

Statistical Analysis

All data are expressed as mean ± SD. Statistical analysis was performed with SPSS Statistics software v. 22 (IBM, NY, USA). Independent-Samples Mann-Whitney U test was used for comparison between two groups. Comparisons of three groups were done with Independent-Samples Kruskal-Wallis test. Correlations between 2 continuous variables were measured with Spearman's rank correlation coefficient (r_s). *P* values less than 0.05 were considered statistically significant.

RESULTS

Immunohistology

Average MI size was 46±6% of the LV circumference (range 40–55) at 7 days and 41±10% (range 22–53) at 4 weeks after coronary ligation. None of the sham-operated rats had MI. Figure 2 shows representative high magnification micrographs of the histological and immunohistochemical findings in the infarcted area. At 7 days, the infarcted area consisted of granulation tissue whereas at 4 weeks, a dense collagenous scar was present. At both 7 days and 4 weeks after MI, there were numerous CD68 positive macrophages and CD31 positive endothelial cells present in the infarcted area.

Immunohistochemistry showed scattered MMP-2 and MMP-9 positive cells in the infarcted area at 7 days after MI. There were only few MMP-2 or MMP-9 positive cells in the remote non-infarcted myocardium. Double staining showed that MMP-9 positivity co-localized often with CD68 positive macrophages, whereas there was no co-localization with α-smooth muscle actin staining in the infarcted area.

Biokinetics and Stability of ^{68}Ga-DOTA-peptide

Radiochemical purity of ^{68}Ga-DOTA-peptide was >95% throughout the study, and specific radioactivity 15±8.6 MBq/nmol. Based on PET/CT imaging, blood concentration of the ^{68}Ga-DOTA-peptide decreased slowly (Figure 3). Radioactivity concentration was higher in the blood than myocardium throughout the imaging periods of 20 or 60 minutes and thus, no specific signal from the infarcted area was visible in the *in vivo* images either at 7 days or 4 weeks after coronary ligation. Blood radioactivity concentration was lower in healthy animals than after either coronary ligation or sham operation (P<0.001, Figure 3). According to radiochromatographical analysis of serial plasma samples , the amount of intact tracer rapidly decreased after injection (Figure 4). At 30 minutes after i.v.injection, 17±1.6 % of plasma total radioactivity was accounting from the intact tracer. Thus, slow blood clearance was mainly due to radiometabolites. Based on *in vivo* tracer biokinetics and stability, time point of 30 minutes post-injection was selected for *ex vivo* analyses.

Biodistribution and Myocardial Uptake of ^{68}Ga-DOTA-Peptide After MI

The results of *ex vivo* biodistribution of ^{68}Ga-DOTA-peptide are shown in Table 1 and Supplementary Fig. 1. The uptake of ^{68}Ga-DOTA-peptide in the LV myocardium was significantly higher in rats with coronary ligation than sham-operation at 7 days and 4 weeks after surgery. Blood radioactivity concentration was comparable between rats with coronary ligation or sham-operation.

The pre-treatment with MMP-2/9 inhibitor reduced tracer binding in the LV myocardium by 24 % (from 0.59 ± 0.07 SUV (n=7) to 0.45 ± 0.033 SUV (n=5), P=0.002). Excessive amount of unlabeled peptide in turn, reduced the tracer uptake in the LV myocardium by 48 % (to 0.30 ±

0.024 (n=3), P<0.001). There were also reductions in other tissues, including plasma, blood, liver, spleen, lungs and skeletal muscle (Supplementary Fig. 1).

The results of ^{68}Ga-DOTA-peptide autoradiography in LV tissue sections are shown in Figure 5 and in Table 2. Tracer uptake was homogeneous and low in the myocardium of sham-operated rats. However, there was a clear, focal increase in the uptake of ^{68}Ga-DOTA-peptide in the infarcted area both at 7 days and 4 weeks after coronary ligation. The average MI-to-remote ratio of ^{68}Ga-DOTA-peptide uptake was 2.5±0.46 (*P*=0.003) at 7 days and 3.1±0.98 (*P*=0.002) at 4 weeks. There were no differences in ^{68}Ga-DOTA-peptide uptake between remote, non-infarcted areas of rats with MI and myocardium of sham-operated either 7 days or 4 weeks after coronary ligation.

There was a strong correlation between the size of MI and the amount of ^{68}Ga-DOTA-peptide uptake in autoradiography images both in the remote and infarcted areas at 7 days after MI (Figure 6). Weaker correlations were observed at 4 weeks (Figure 6).

There was a correlation between the uptake of ^{68}Ga-DOTA-peptide in autoradiography images and areal percentage of CD68 positive macrophages in the infarcted area at 7 days after MI (Figure 7). Tracer uptake also correlated with CD31 positive area both at 7 days and 4 weeks after MI. However, there were no correlations between the uptake of ^{68}Ga-DOTA-peptide and areal percentage of collagen in the infarcted area. Tracer uptake was low in the remote, non-infarcted areas and thus, no correlations to histology were seen.

DISCUSSION

We found that ^{68}Ga-DOTA-peptide targeting MMP-2/9 expression is taken up in the infarcted myocardium after recent ischemic injury or in the presence of an infarct scar in rat. Pre-treatment with MMP-2/9 inhibitor or excess of unlabeled peptide significantly reduced tracer uptake indicating specific binding. In the infarcted area, tracer uptake correlated with

macrophages and endothelial cells. Double staining showed co-localization of MMP-9 with CD68 positive macrophages, but both MMP-9 and MMP-2 have also been previously shown to be involved in post-ischemic neovascularization[17] after ischemic myocardial injury[7,18,19,]. The tracer was rapidly metabolized, radioactivity in the blood remained high and tracer uptake in the myocardium was not detectable by *in vivo* PET.

MMP-2 and MMP-9 are potentially good targets for imaging: MMP-9 is synthesized as an inactive pro-enzyme and transported in specialized, kinesin-associated vesicles to the cell surface, where it stays associated with the cell membrane or becomes secreted[16]. To our knowledge, this is the first study to test PET tracer for imaging their cardiac expression. Although ex vivo analyses demonstrated uptake of MMP-2/9 targeting ^{68}Ga-DOTA-peptide in the infarcted myocardium, *in vivo* imaging of tracer uptake was not feasible due to low myocardium-to-blood ratio. This was most likely related to limitations in tracer properties, i.e. slow blood clearance and rapid metabolism as well as relatively low myocardial uptake. In the *in vivo* images, higher activity in the non-infarcted intervenricular septum than the infarcted area (Figure 3) as well as gradual reduction of myocardial tracer uptake is most likely related to spill over from the right or left ventricle cavity blood pool into the myocardial ROI. Spillover cannot be avoided due to limited spatial resolution of imaging and relatively high blood activity.

The reduction of tracer uptake by pre-treatment with MMP-2/9 inhibitor or unlabeled peptide indicates specific uptake in the myocardium. However, myocardial autoradiography signal could also be partly attributed to non-specific tissue uptake due to residual radiometabolites in the blood pool. A limitation of our study is that the protocol did not include direct detection of MMP-2/9 activation in the myocardium by zymography and thus, we are not able to confirm specificity of the tracer towards the activated enzyme. Previously, the peptide has shown high selectivity towards gelatinases as compared with other MMP types[12].

Autoradiography showed increased tracer uptake in the infarcted area as compared to the remote myocardium or myocardium of sham- operated rats at 1 week and 4 weeks after MI. This is consistent with localization and time course of MMP-2/9 expression after MI in previous studies[5,10,19] that have highlighted differences in the regional and temporal activation patterns of MMPs after MI[5]. The importance of MMP-2/9 expression has been highlighted by the decreased rate of post-MI cardiac rupture and the attenuation of adverse LV remodeling in gene knockout animals lacking MMP-2/9 expression[8,9], as well as association to impaired regional myocardial systolic strain[5].

New tracers for imaging activation of selective MMP types are being actively developed. An example is a dual-isotope (^{177}Lu/^{125}I) radiolabeled activatable cell-penetrating peptide probe (ACPP) that is sensitive to the proteolytic activity of MMP-2/9 and subsequently becomes trapped in tissue[20]. This kind of molecular imaging probe could be an option to amplify the imaging signal intensity.

NEW KNOWLEDGE GAINED

The ^{68}Ga-labeled PET tracer targeting MMP-2/9 accumulated in the myocardium after ischemic injury, but the signal was not detectable by *in vivo* PET.

CONCLUSIONS

MMP-2/9 targeting ^{68}Ga-DOTA-peptide accumulates in the damaged rat myocardium after an ischemic injury, but tracer instability and slow clearance *in vivo* makes it unsuitable for further evaluation.

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Disclosure

The authors have no conflicts of interests to report.

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Table 1. Biodistribution of ^{68}Ga-DOTA-peptide targeting MMP-2/9 expression 30 minutes after injection in rats at 7 days (7d) or 4 weeks (4w) after myocardial infarction (MI) or sham–operation as measured *ex vivo* by gamma counting of tissue samples

	7d Sham	7d MI	*P* (7d Sham vs. 7d MI)	4w Sham	4w MI	*P* (4w Sham vs. 4w MI)
Left Ventricle	0.40±0.13	0.59±0.072	0.030	0.39±0.067	0.58±0.14	0.019
Blood	1.2±0.34	1.26±0.31	1.0	1.1±0.23	1.2±0.24	0.79
Plasma	2.1±0.55	2.1±0.52	1.0	2.1±0.45	2.3±0.58	0.79
Thymus	0.20±0.049	0.23±0.059	0.39	0.20±0.025	0.23±0.085	1.0
Liver	1.4±0.19	0.75±0.32	0.071	1.5±0.33	1.2±0.58	0.38
Kidneys	3.6±2.8	3.9±1.2	1.0	3.3±0.49	3.7±1.80	0.86
Spleen	1.4±0.25	0.97±0.48	0.267	1.3±0.21	1.1±0.54	0.86
Lungs	1.0±0.021	0.81±0.26	0.143	1.4±0.10	1.9±0.58	1.0
Skeletal Muscle	0.15±0.045	0.17±0.020	0.432	0.13±0.020	0.17±0.11	0.54
Urine	230±130	270±160	0.88	190±180	120±37	0.004

The results are shown as standardized uptake value (SUV, mean±SD with two significant figures).

Table 2. Uptake of ^{68}Ga-DOTA-peptide in the infarcted area (MI) and remote non-infarcted area of the rat myocardium at 7 days (7d) or 4 weeks (4w) after coronary ligation or sham-operation as determined by autoradiography of tissue cryosections

	7d Sham	7d MI	4w Sham	4w MI
MI	-	29±2.8[a]	-	31±5.0[c]
Remote	12±3.4	12±3.0[b]	11±2.4	11±4.6[d]

The results are expressed as photostimulated luminescence/mm^2 (PSL/mm^2, mean±SD). [a]=p=0.003 vs. Remote, [b]=p=0.88 vs. Sham, [c]=p=0.002 vs. Remote, [d]=p=0.72 vs. Sham

FIGURE LEGENDS

Figure 1. Structure of ^{68}Ga-DOTA-peptide tracer (Cys$^{3-10}$; H-Gly-Ala-Cys-Leu-Arg-Ser-Gly-Arg-Gly-Cys-Gly-PEG(3)-DOTA-^{68}Ga).

Figure 2. Histology and immunohistochemistry of rat myocardium after coronary ligation or sham-operation. Micrographs show Masson's trichrome stained left ventricular myocardium of a sham-operated animal (**a**) and infarcted area at 7 days (**b**) and 4 weeks (**c**) after coronary ligation. Myocytes appear red and collagen fibers blue. Micrographs of tissue sections from the infarcted area 7 days after ligation stained with immunohistochemistry show MMP-2 positive cells in remote (**d**) and infarcted area (**e**), MMP-9 positive cells in remote (**f**) and infarcted area (**g**), CD31 positive endothelial cells (**h**), and CD68 positive macrophages (**i**),in brown color. Double staining shows MMP-9 positivity in brown color and either α smooth muscle actin (α-SMA) in fibroblasts or smooth muscle cells (**j**) or CD68 staining of macrophages as blue color (**k**). Note that MMP-9 co-localizes with many CD68 positive cells (arrows). In contrast, MMP-9 does not co-localize with α-SMA positive cells. Scale bars are 50 μm.

Figure 3. *In vivo* biokinetics of ^{68}Ga-DOTA-peptide in rats. Time-activity curves of sham-operated rats (**a**, n=5), blood of healthy, non-operated animals (**b**, n=3), or rats with myocardial infarction (MI) either 7 days (**c**, n=3) or 4 weeks (**c**, n=7 for 20 min and n=3 for 60 min PET imaging) after coronary ligation. Transaxial PET/CT images show tracer uptake in different time points (CT reference **d** , 0-2 min **e**, 2-10 min **f**, 10-60 min **g**) 4 weeks after myocardial infarction.

Figure 4. *In vivo* stability of intravenously administered ^{68}Ga-DOTA-peptide in rat plasma at 4 weeks after coronary ligation (**a**). At 30 min post-injection, two radioactive metabolites were detected in plasma with radio-HPLC analysis (**b**).

Figure 5. Distribution of^{68}Ga-DOTA-peptide in rat myocardium as detected by autoradiography. Micrographs of cross sections of the left ventricle stained with Masson's trichrome after sham-

operation (**a**) and either 7 days (**c**) or 4 weeks (**e**) after coronary ligation. Autoradiographs of the same sections show homogeneous, low uptake of ^{68}Ga-DOTA-peptide in the myocardium of sham-operated rat (**b**) and in the remote, non-infarcted myocardium of rats with coronary ligation (**d** and **f**). Note that there is focally increased uptake of ^{68}Ga-DOTA-peptide co-localizing with the infarcted areas (arrows in **c**-**f**) both at 7 days (**d**) and 4 weeks (**f**) after MI. Scale bar is 5 mm.

Figure 6. Correlations between ^{68}Ga-DOTA-peptide uptake in autoradiography and the size of myocardial infarction (MI) at 7 days (**a** and **b**) after coronary ligation or 4 weeks (**c** and **d**) after coronary ligation in the remote (**a** and **c**) and infarcted (**b** and **d**) areas. r_s, Spearman's correlation coefficient; *PSL*, photostimulated luminescence; *circ-%*, percentage of infarcted LV circumference.

Figure 7. Scatter plots show correlations between ^{68}Ga-DOTA-peptide uptake and the areal percentages of CD68 positive macrophages in infarcted area at 7 days (**a**) or 4 weeks (**b**) as well as CD31 staining of endothelial cells in infarcted area at 7 days (**c**) or 4 weeks (**d**) after coronary ligation. r_s, Spearman's correlation coefficient; *PSL*, photostimulated luminescence.

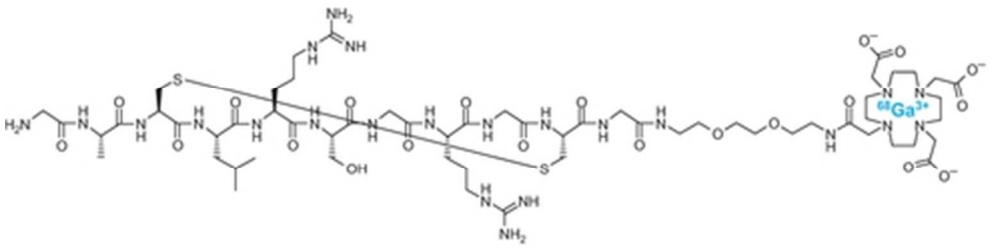

Figure 1. Structure of ^{68}Ga-DOTA-peptide tracer (Cys$^{3-10}$; H-Gly-Ala-Cys-Leu-Arg-Ser-Gly-Arg-Gly-Cys-Gly-PEG(3)-DOTA-^{68}Ga)
Figure 1
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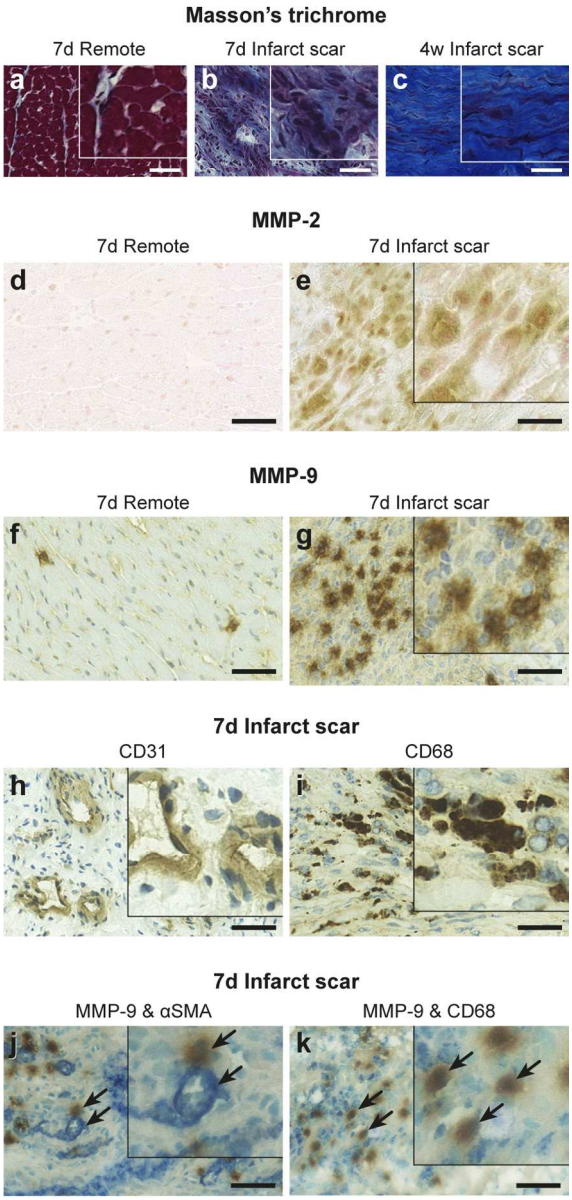

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Figure 2
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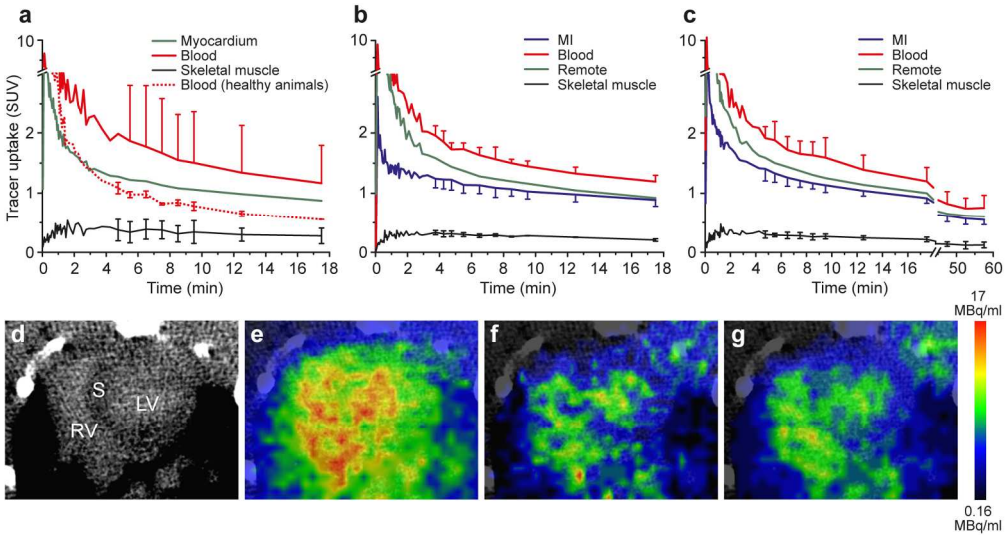

Figure 3. *In vivo* biokinetics of ^{68}Ga-DOTA-peptide in rats. Time-activity curves of sham-operated rats (**a**, *n*=5), blood of healthy, non-operated animals (**b**, *n*=3), or rats with myocardial infarction (MI) either 7 days (**c**, *n*=3) or 4 weeks (**c**, *n*=7 for 20 min and *n*=3 for 60 min PET imaging) after coronary ligation. Transaxial PET/CT images show tracer uptake in different time points (CT reference **d** , 0-2 min **e**, 2-10 min **f**, 10-60 min **g**) 4 weeks after myocardial infarction.

Figure 3
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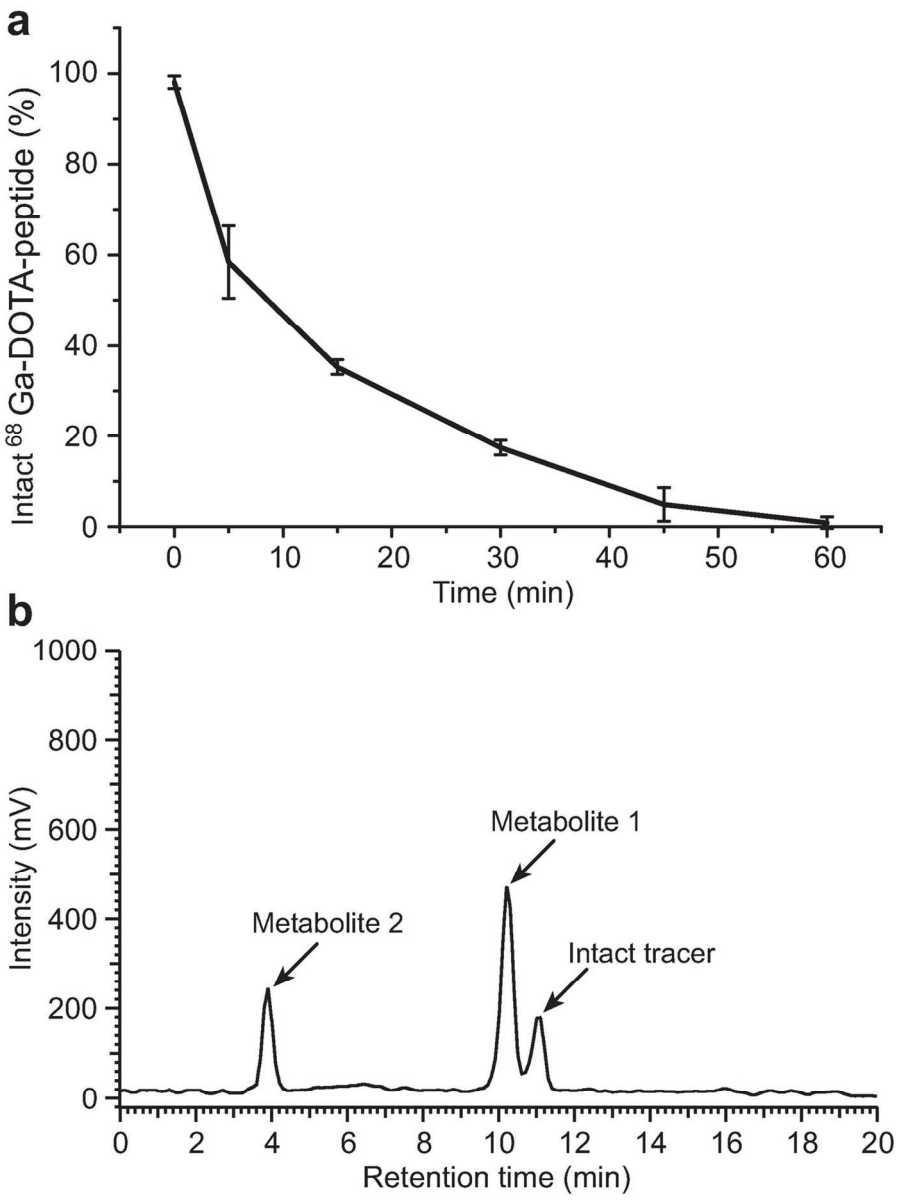

Figure 4. *In vivo* stability of intravenously administered ^{68}Ga-DOTA-peptide in rat plasma at 4 weeks after coronary ligation (**a**). At 30 min post-injection, two radioactive metabolites were detected in plasma with radio-HPLC analysis (**b**).

Figure 4
113x150mm (300 x 300 DPI)

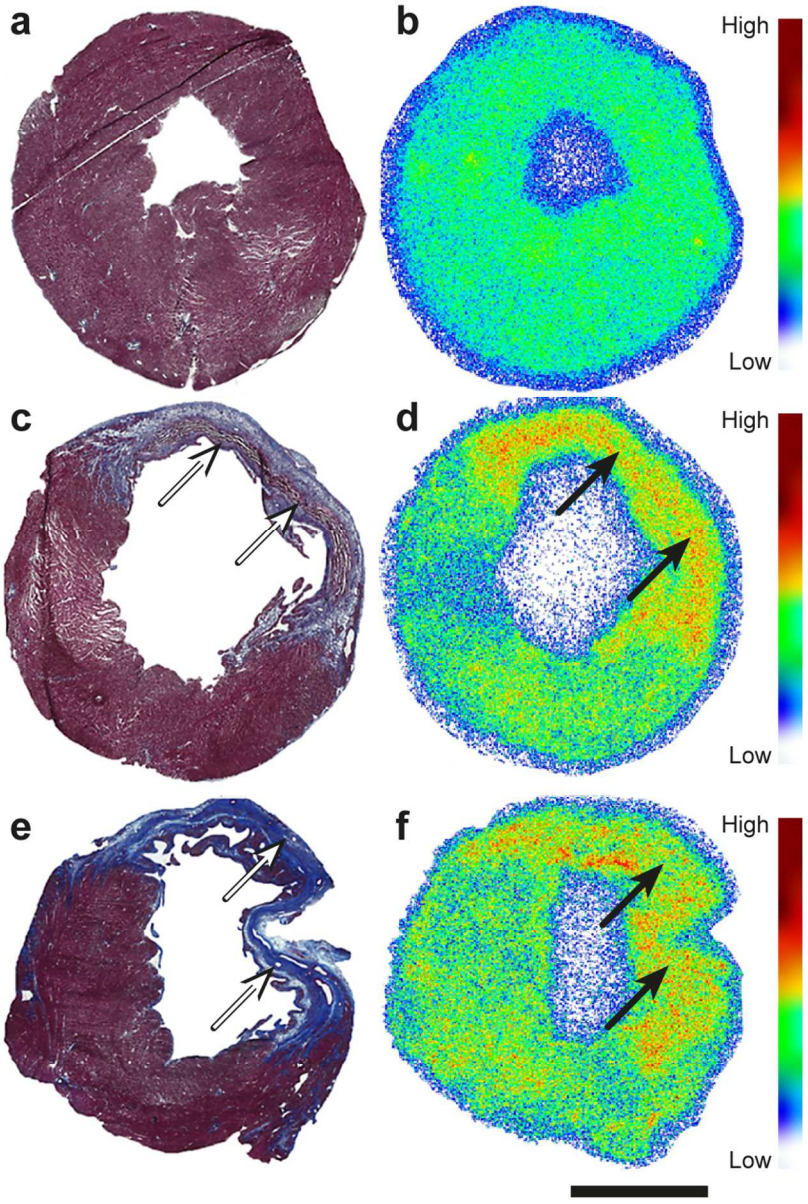

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Figure 5
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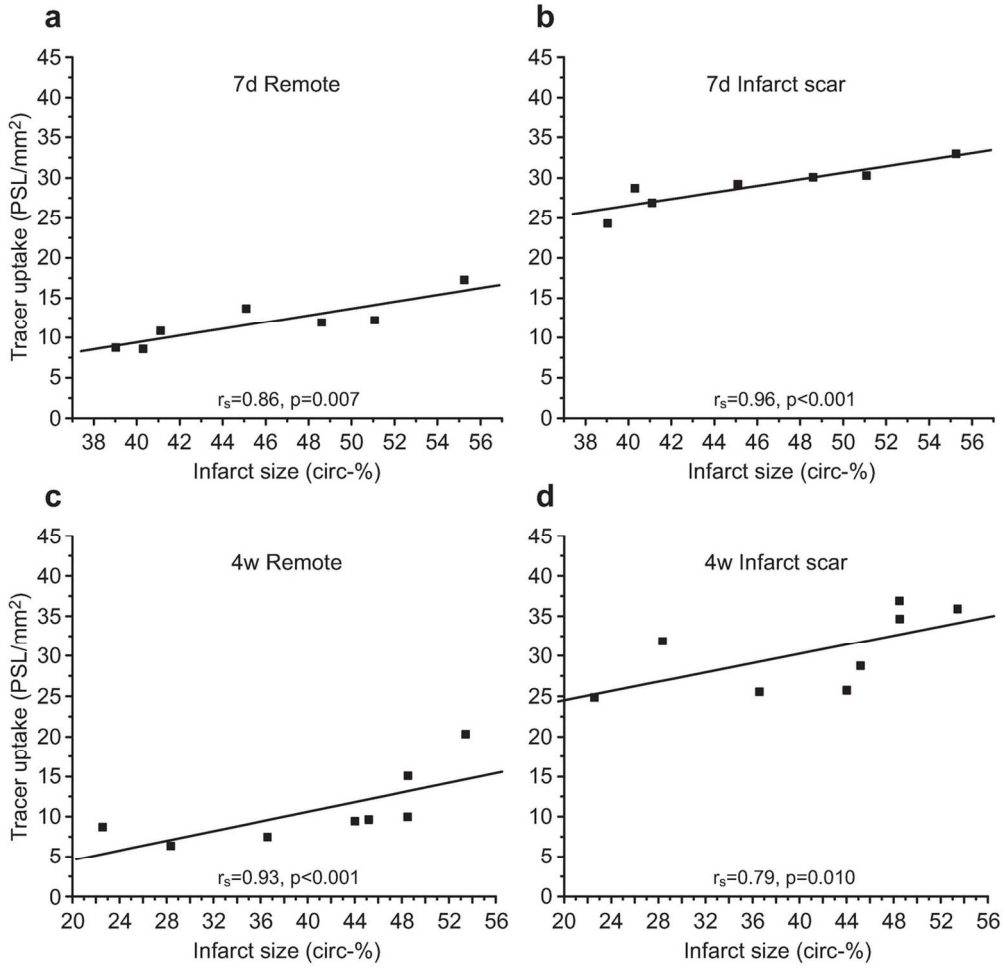

Figure 6. Correlations between ^{68}Ga-DOTA-peptide uptake in autoradiography and the size of myocardial infarction (MI) at 7 days (**a** and **b**) after coronary ligation or 4 weeks (**c** and **d**) after coronary ligation in the remote (**a** and **c**) and infarcted (**b** and **d**) areas. r_s, Spearman's correlation coefficient; *PSL*, photostimulated luminescence; *circ-%*, percentage of infarcted LV circumference.

Figure 6
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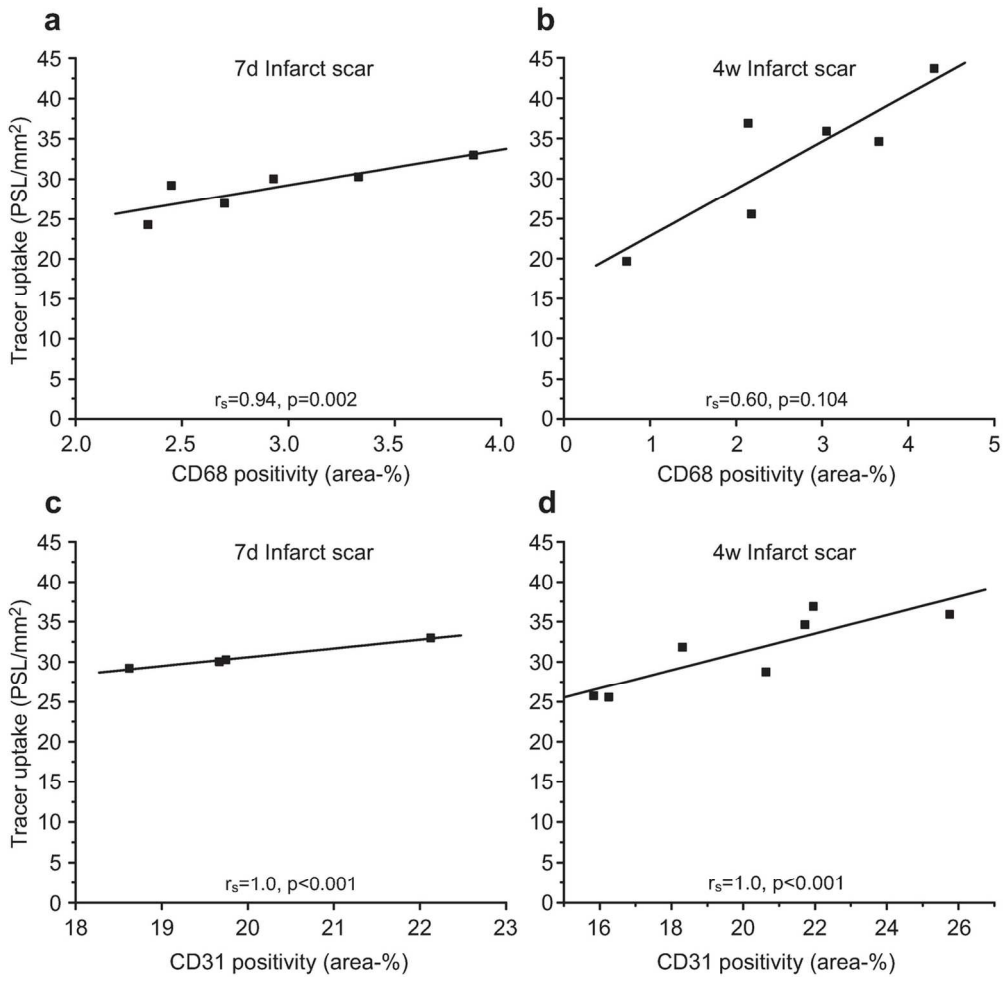

Figure 7. Scatter plots show correlations between ^{68}Ga-DOTA-peptide uptake and the areal percentages of CD68 positive macrophages in infarcted area at 7 days (**a**) or 4 weeks (**b**) as well as CD31 staining of endothelial cells in infarcted area at 7 days (**c**) or 4 weeks (**d**) after coronary ligation. r_s, Spearman's correlation coefficient; *PSL*, photostimulated luminescence.

Figure 7
124x121mm (300 x 300 DPI)

ELECTRONIC SUPPLEMENTARY MATERIAL

Evaluation of ^{68}Ga-Labeled Peptide Tracer for Detection of Gelatinase Expression after Myocardial Infarction in Rat

Max Kiugel, BM, MSc[1], Ville Kytö, MD, PhD[2,3], Tiina Saanijoki, MSc[1], Heidi Liljenbäck, MSc[1,4], Olli Metsälä, BSc[1], Mia Ståhle, MSc[1], Johanna Tuomela, PhD[5], Xiang-Guo Li, PhD[1,6], Pekka Saukko, MD, PhD[7], Juhani Knuuti, MD, PhD[1,8], Anne Roivainen, PhD[1,4,8], Antti Saraste, MD, PhD[1,8,9]

[1]Turku PET Centre, University of Turku, Turku, Finland; [2]Heart Center, Turku University Hospital, Turku, Finland; [3]Research Centre of Applied and Preventive Cardiovascular Medicine, University of Turku, Turku, Finland; [4]Turku Center for Disease Modeling, University of Turku, Turku Finland; [5]Department of Cell Biology and Anatomy, University of Turku, Turku, Finland; [6]Turku PET Centre, Åbo Akademi University, Turku, Finland; [7]Department of Pathology and Forensic Medicine, University of Turku, Turku, Finland; [8]Turku PET Centre, Turku University Hospital, Turku, Finland; [9]Institute of Clinical Medicine, University of Turku, Turku, Finland

MATERIALS AND METHODS

Radiochemistry

The 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-conjugated peptide (Figure 1) was purchased from Peptide Specialty Laboratories GmBH (Heidelberg, Germany) and ^{68}Ga-labeled as previously described[1]. Briefly, ^{68}Ga was obtained from a ^{68}Ge/^{68}Ga generator (Eckert & Ziegler, California, USA) by elution with 0.1 mol/L HCl made from ultrapure 30% HCl and ultrapure water. ^{68}Ga eluate (0.5 mL) was mixed with sodium acetate (18 mg) to give a pH of approximately 5.5. Next, 14 nmol (22 µg) DOTA-peptide (dissolved in deionized water to give stock solution of 1 mmol/L) was added, and the reaction mixture was heated at 95°C for 20 min. No further purification was performed. Radiochemical purity of ^{68}Ga-DOTA-peptide was determined by flow-through radiodetector coupled reversed-phase high-performance liquid chromatography (radio-HPLC; Jupiter C18 column, 4.6×150 mm, 300 Å, 5 µm; Phenomenex, Torrance, CA, USA). The HPLC conditions were as follows: flow rate=1 mL/min; λ=215 nm; A=0.1% trifluoroacetic acid (TFA)/water; B=0.1% TFA/acetonitrile. A/B gradient: 0–5 min, 97/3; 5–15 min, from 97/3 to 0/100. The radio-HPLC system consisted of LaChrom Instruments (Hitachi; Merck, Darmstadt, Germany) and of a Radiomatic 150TR radioisotope detector (Packard, Meriden, CT, USA).

In Vivo Stability of Tracer

The *in vivo* stability of the ^{68}Ga-DOTA-peptide was studied in 2 rats at 4 weeks after coronary ligation until 60 minutes post injection of ^{68}Ga-DOTA- peptide and blood samples were obtained at 5, 15, 30, 45 and 60 min after injection. The plasma was separated by centrifugation and the proteins of plasma were precipitated with acetonitrile (5:7, *v*:*v*). Supernatant obtained after centrifugation was filtered through 0.45-µm Minispike filter (Waters Corporation) and analyzed by radio-HPLC (Jupiter C18, 10×250 mm, 300 Å, 5 µm; Phenomenex, Torrance, CA, USA) The

HPLC conditions were as follows: flow rate=5 mL/min; λ=215 nm; A=0.1% TFA/water; B=0.1% TFA/acetonitrile. A/B gradient: 0–5 min, 97/3; 5–15 min, from 97/3 to 0/100.

PET/CT Acquisition

The rats were imaged using a dedicated small-animal PET/CT (Inveon Multimodality; Siemens Medical Solutions, Knoxville, TN, USA) under isoflurane anesthesia (3.0% for induction and 1.8% for maintenance). The electrocardiogram was monitored and body temperature was maintained using a heating pad throughout the imaging. PET data were acquired for 20 or 60 min starting at the time of injection of ^{68}Ga-DOTA-peptide and were sorted into 30×3 s, 9×10 s, 4×30 s, 5×60 s and 10×300 s time frames. The images were reconstructed using ordered-subset expectation maximization algorithm with 4 iterations. Immediately after PET, 200-µL of intravascular iodinated contrast agent eXIA^{TM}160XL (Binitio Biomedical Inc, Ottawa, ON, Canada) was injected and high-resolution CT was acquired. CT acquisition consisted of 160 projections with the exposure time of 1250 ms, x-ray voltage of 80 kV, and anode current of 500 µA for a 220° rotation. CT images were reconstructed using filtered back-projection algorithm.

Autoradiography

The LV was frozen in isopentane and sliced into serial of 8- and 20-µm transverse cryosections from apex to base (five to six intervals per heart) for analysis of ^{68}Ga-DOTA-peptide uptake by digital autoradiography as previously described[2]. Briefly, air-dried sections were apposed to an imaging plate for >2 radionuclide half-lives (Fuji Imaging Plate BAS-TR2025, Fuji Photo Film Co. Tokyo, Japan) and scanned with Fuji BAS-5000 analyzer (Fuji Photo Film Co. Tokyo, Japan; internal spatial resolution of 25-µm)[3]. Tracer accumulation was measured with TINA software v. 2.1 (Raytest Isotopenmessgeräte, GmbH, Straubenhardt, Germany) as count density (photostimulated luminescence per square millimeter, PSL/mm^2). The background area count

densities were subtracted from the image data, which was then normalized for injected radioactivity dose, decay and exposure time of the imaging plate. Radioactivity accumulation was measured in 20-µm sections, (3-5 from apex to base where the infarct scar was present), which provided better count statistics than the 8-µm sections. Autoradiographs were co-registered with images of the same tissue sections stained with hematoxylin and eosin (HE). Exact location of the infarct scar was also confirmed by adjacent sections stained with Masson's trichrome. Based on histology, ROIs were defined as the infarcted area (covering the whole infarcted region) and the remote, non-infarcted area in the posterior or inferior wall of the LV.

References

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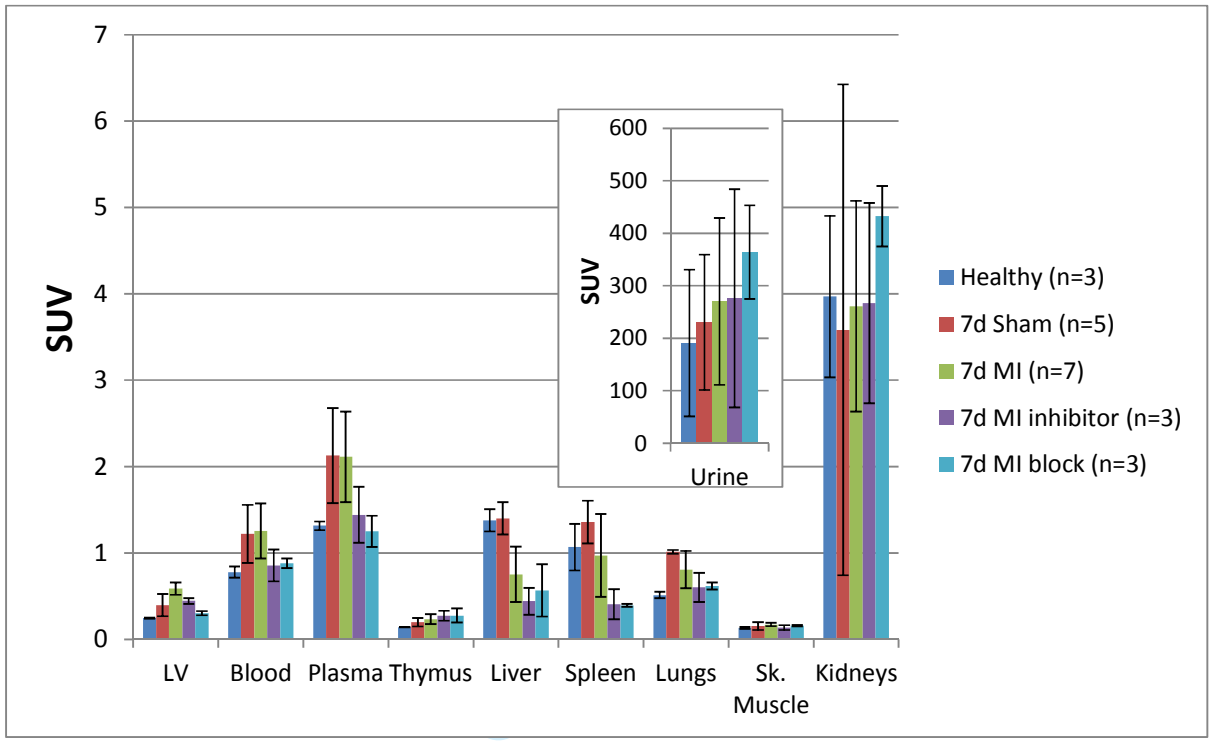

Supplementary Figure 1. Biodistribution of MMP-2/9 activation targeting ^{68}Ga-DOTA-peptide 30 minutes after injection in rats at 7 days (7d) after myocardial infarction (MI) or sham–operation (Sham) as measured *ex vivo* by gamma counting of tissue samples. *Inhibitor*, pre-treatment with MMP-2/9 inhibitor before injection of ^{68}Ga-DOTA-peptide. *Block*, pre-treatment with excess of unlabelled peptide, *LV*, left ventricle.

Evaluation of ^{68}Ga-Labeled Peptide Tracer for Detection of Gelatinase Expression after Myocardial Infarction in Rat

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Main text (Abstract-Figure Legends) 4430 words; abstract 223 words

Conflict of interest: All the authors declare that they have no conflicts of interest.

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Abstract

Background. Matrix metalloproteinases 2 and 9 (MMP-2/9) play a role in extracellular matrix remodeling after an ischemic myocardial injury. We evaluated ^{68}Ga-DOTA-peptide targeting MMP-2/9 for the detection of gelatinase expression after myocardial infarction (MI) in rat.

Methods. Rats were injected with 43 ± 7.7 MBq of ^{68}Ga-DOTA-peptide targeting MMP-2/9 at 7 days (*n*=7) or 4 weeks (*n*=8) after permanent coronary ligation or sham operation (n=5 at both time-points) followed by positron emission tomography (PET). The left ventricle was cut in frozen sections for autoradiography and immunohistochemistry 30 minutes after tracer injection.

Results. Immunohistochemical staining showed MMP-2 and MMP-9 expressing cells, CD31 positive endothelial cells, and CD68 positive macrophages in the infarcted myocardium. Autoradiography showed increased tracer uptake in the infarcted area both at 7 days and 4 weeks after MI (MI-to-remote area ratio 2.5±0.46 and 3.1±1.0, respectively). Tracer uptake in damaged tissue correlated with the amount of CD68 positive macrophages at 7 days after MI, and CD31 positive endothelial cells at 7 days and 4 weeks after MI. The tracer was rapidly metabolized, radioactivity in the blood exceeded that of the myocardium and tracer accumulation in the heart was not detectable by *in vivo* PET.

Conclusions. ^{68}Ga-DOTA-peptide targeting MMP-2/9 accumulates in the damaged rat myocardium after an ischemic injury, but tracer instability and slow clearance *in vivo* makes it unsuitable ~~inapplicable~~ for further evaluation.

Key Words: matrix metalloproteinase, myocardial infarction, positron emission tomography

Abbreviations

DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
ECM	Extracellular matrix
LCA	Left coronary artery
LV	Left ventricle
MI	Myocardial infarction
MMP	Matrix metalloproteinase
PET/CT	Positron emission tomography/computed tomography
PSL	Photostimulated luminescence
ROI	Region of interest
SUV	Standardized uptake value

INTRODUCTION

Matrix metalloproteinases (MMPs) are proteolytic enzymes that play a central role in the degradation of extracellular matrix (ECM) proteins in ~~associated with~~ myocardial infarction (MI), healing process, and subsequent left ventricle (LV) remodeling[1,2,3]. Different MMP types have diverse spatial and temporal activation patterns in the heart after MI[3,4,5]. In particular, the expression of gelatinases MMP-2 and MMP-9 is elevated after an ischemic myocardial injury[4,5,6] in association with the early injury responses, such as inflammation and neovascularization[1,7], as well as post-MI remodeling[8,9]. While MMP-9 is activated during the first days after an ischemic injury, MMP-2 activation occurs later and persists longer during the post-MI remodeling phase[4,10].

Molecular imaging facilitates *in vivo* evaluation of MMP activation within the myocardium after MI[5,6,10]. Chen and co-workers demonstrated the feasibility of detecting MMP activity after MI using a near-infrared fluorescent probe that is activated upon cleavage by MMP-2/9[10]. Thereafter, radiolabeled broad-spectrum MMP-inhibitor has been used to visualize MMP activation after MI with the single-photon emission computed tomography (SPECT)[5,6]. Given the differences in activation patterns and functions of different MMP types within the post-MI myocardium, methods to assess expression of specific MMPs would be warranted.

In this study we have used a previously developed MMP-2/9 targeting positron emission tomography (PET) imaging agent (Figure 1)[11]. The peptide was identified from phage display library ~~display~~ and selected based on its ability to bind chemically activated MMP-9[11,12] and shown to inhibit both MMP-9 and MMP-2[12] . The ^{68}Ga-labeled peptide tracer provides good target-to-background ratio for PET imaging of tumor xenografts expressing MMP-9 in rats[11]. Purpose of this study was to evaluate the feasibility of ^{68}Ga-labeled 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid conjugated MMP-2/9 targeting tracer (^{68}Ga-DOTA-peptide) for studying the distribution and extent of gelatinase expression during MI healing process and post-MI LV remodeling. Immunohistochemistry was used to detect MMP-9

expressing cells in the myocardium together with other biological processes associated with ECM remodeling following acute MI. Biodistribution and myocardial uptake of the MMP-2/9 targeting ^{68}Ga-DOTA-peptide were studied by *in vivo* PET/computed tomography (CT) as well as radioactivity measurements and autoradiography of tissue samples. We hypothesized that with the specific probe we could monitor myocardial injury responses involving MMP-2/9 during early healing and remodeling phases after MI.

MATERIALS AND METHODS

Animal Model and Study Design

MI was induced by permanent surgical ligation of the left coronary artery (LCA) according to previously described procedures[13]. Briefly 0.2 mg/kg of buprenorphine (Temgesic; Schering-Plough, Espoo, Finland) was administered intramuscularly prior to operation for analgesia, the rats were anesthetized with a combination of inhaled isoflurane (Vet Medic Animal Health, Parola, Finland) (induction only) and subcutaneous injection of 10 mg/kg of xylazine (Rompun; Orion Pharma, Espoo, Finland) and 90 mg/kg of ketamine (Ketaminol; Orion Pharma, Espoo, Finland), intubated, connected to a respirator, thoracotomy was performed and suture was placed in the proximal LCA. The sham operation consisted of the same procedures except coronary ligation. A total of 65 male Sprague-Dawley rats ageing 7±1 weeks and weighing 330±71 g were used. Mortality was approximately 25% after either coronary ligation or sham-operation and occurred during the first two days after surgery. Animals that did not develop MI (scar < 4% of the LV circumference) were excluded from the study.

The main study group for evaluation of myocardial uptake of ^{68}Ga-DOTA-peptide consisted of 7 rats studied at 7 days and 8 rats studied at 4 weeks after coronary ligation; and 5 rats studied at 1 week and 5 rats at 4 weeks after the sham-operation. 3 unoperated rats were also used as controls. Rats were intravenously (i.v.) injected with 43±7.7 MBq (6.9±3.7 nmol) of ^{68}Ga-DOTA-

peptide via tail vein and imaged by dynamic PET/CT for 20 minutes starting at the time of injection. Rats were killed 30 minutes post-injection by cervical dislocation and tissue samples were obtained for measurement of tracer uptake by gamma counter or autoradiography as well as for histology.

In order to assess the specificity of ^{68}Ga-DOTA-peptide accumulation in the heart 7 days after coronary ligation, 5 rats were i.v. injected with ≥1.4 µmol/kg (approximately ≥20 µmol/L of blood volume, IC_{50}=10 µmol/L) of specific MMP-2/9 inhibitor [H-Cys^{1}-Thr-Thr-His-Trp-Gly-Phe-Thr-Leu-Cys^{10}-OH (cyclic: 1→10)] (product number 444251, Merck KGaA, Darmstadt, Germany) 5 minutes prior to the administration of ^{68}Ga-DOTA-peptide. In addition, 3 rats were injected with ~500 fold amount (compared to administrated ^{68}Ga-DOTA-peptide), of unlabeled peptide. ~~Seven rats with 7d MI were used as controls.~~

Tracer biokinetics were evaluated for a longer period of time by *in vivo*, i.e. a 60-min dynamic PET/CT scan was performed for 3 rats at 4 weeks after coronary ligation, 1 sham-operated rat, and 3 healthy control rats. These rats were i.v. injected with 42±6.7 MBq (15±8.6 nmol) of ^{68}Ga-DOTA-peptide.

The study protocol was approved by the National Animal Experiment Board in Finland and the Regional State Administrative Agency for Southern Finland, and carried out in compliance with the relevant European Union directives.

Radiochemistry and *In Vivo* Stability

The DOTA-conjugated peptide (Figure 1) was purchased from Peptide Specialty Laboratories GmBH (Heidelberg, Germany) and ^{68}Ga labelled as previously described[11]. The *in vivo* stability of the ^{68}Ga-DOTA-peptide was studied in 2 rats at 4 weeks after coronary ligation until 60 minutes post injection of 49±3.8 MBq. Detailed description of labeling and analyses are described in the electronic supplementary material.

PET/CT Image Analysis

The rats were imaged using a small-animal PET/CT (Inveon Multimodality; Siemens Medical Solutions, Knoxville, TN, USA) under isoflurane anesthesia. The full width at half maximum resolution of the scanner for ^{68}Ga is 2.46 mm.[14] PET data was acquired for 20 or 60 min starting at the time of injection of ^{68}Ga-DOTA-peptide. Immediately after PET, 200-µL of intravascular iodinated contrast agent eXIA^{TM}160XL (Binitio Biomedical Inc, Ottawa, ON, Canada) was injected i.v. and high-resolution CT was acquired. Detailed protocols are described in the supplement. Images were analysed using Carimas v.2.6 software (Turku PET Centre, Turku, Finland). Alignment of PET and CT images was automatic and confirmed by anatomical landmarks. Data was normalized and corrected for injected radioactivity dose and radionuclide decay. Regions of interest (ROIs) were drawn according to high-resolution CT image in the infarcted myocardium (or corresponding location in the anterior wall in sham operated rats), remote myocardium in the septum, blood pool (inside the LV cavity), and skeletal muscle. Results were reported as mean radioactivity concentration (Bq/mL converted to standardized uptake values [SUV]) as a function of time after injection, i.e. as time-activity curves.

***Ex Vivo* Biodistribution**

A blood sample was obtained by cardiac puncture. Then, the LV (rinsed with saline and without atria or the right ventricle) and various other tissues were excised, weighed and measured for radioactivity using a gamma counter (Triathler 3", Hidex, Turku, Finland). Results were expressed as SUV ~~normalized for injected radioactivity dose, decay time between injection and measurement, animal weight, and the weight of tissue sample~~.

Autoradiography

The LV was frozen in isopentane and sliced into serial 8- and 20-µm transverse cryosections from apex to base for analysis of ^{68}Ga-DOTA-peptide uptake by digital autoradiography as previously described[13]. Radioactivity accumulation was measured in 20-µm sections, which provided better count statistics. Autoradiographs were co-registered with images of the same tissue sections stained with hematoxylin and eosin (HE). Based on histology, ROIs were defined as the infarcted area (covering the whole infarcted region) and the remote, non-infarcted area in the posterior or inferior wall of the LV. For more details see electronic supplementary material.

Histology and Immunohistochemistry

Serial LV cryosections were hematoxylin and eosin (HE) stained for general histology. Masson's trichrome (Sigma-Aldrich, St. Louis, MO, USA) staining was used to distinguish fibrosis and collagen from cardiomyocytes. Macrophages, endothelial cells, MMP-2 and MMP-9 positive cells were detected by immunohistochemical staining using the following primary antibodies: mouse monoclonal anti-rat CD68 (dilution 1:10000, Product N:o MCA341GA, AbD Serotec, Munich, Germany), monoclonal anti-rat CD31 (dilution 1:10000, Product N:o MCA1334GA, AbD Serotec), polyclonal anti-MMP-2 (dilution 1:100, Product N:o ab19167, Merck, Darmstadt, Germany) and polyclonal anti-MMP-9 (dilution 1:1000, Product N:o ab38898, Abcam, Cambridge, UK). Double stainings with anti-MMP-9 (1:1000) and either CD68 (1:15000) or monoclonal antibody for α-smooth muscle actin (1:10000, Product N:o A5228, Sigma Aldrich) were performed in several sections to identify cell types expressing MMP-9.

Digital images of the stained sections were captured with 3D Histech Pannoramic 250 Flash digital slide scanner (3D Histech, Budapest, Hungary). Size of the MI was measured in Masson's trichrome stained sections with Image-J v. 1.46 software (National Institutes of Health, Bethesda, MD) as circumferential percentage of the infarct scar of the whole endocardial length of the LV. Furthermore, percentages of myocardium positive for CD68 or CD31 or collagen (blue color in

Masson's trichrome staining) were quantified within the infarcted and remote areas with the use of Image-J software and specific color thresholds.

Statistical Analysis

All data are expressed as mean ± SD. Statistical analysis was performed with SPSS Statistics software v. 22 (IBM, NY, USA). Independent-Samples Mann-Whitney U test was used for comparison between two groups. Comparisons of three groups were done with Independent-Samples Kruskal-Wallis test. Correlations between 2 continuous variables were measured with Spearman's rank correlation coefficient (r_s). *P* values less than 0.05 were considered statistically significant.

RESULTS

Immunohistology

Average MI size was 46±6% of the LV circumference (range 40–55) at 7 days and 41±10% (range 22–53) at 4 weeks after coronary ligation. None of the sham-operated rats had MI. Figure 2 shows representative high magnification micrographs of the histological and immunohistochemical findings in the infarcted area. At 7 days, the infarcted area consisted of granulation tissue whereas at 4 weeks, a dense collagenous scar was present. At both 7 days and 4 weeks after MI, there were numerous CD68 positive macrophages and CD31 positive endothelial cells present in the infarcted area.

Immunohistochemistry showed scattered MMP-2 and MMP-9 positive cells in the infarcted area at 7 days after MI. There were only few MMP-2 or MMP-9 positive cells in the remote non-infarcted myocardium. Double staining showed that MMP-9 positivity co-localized often with CD68 positive macrophages, whereas there was no co-localization with α-smooth muscle actin staining in the infarcted area.

Biokinetics and Stability of ^{68}Ga-DOTA-peptide

Radiochemical purity of ^{68}Ga-DOTA-peptide was >95% throughout the study, and specific radioactivity 15±8.6 MBq/nmol. Based on PET/CT imaging, blood concentration of the ^{68}Ga-DOTA-peptide decreased slowly (Figure 3). Radioactivity concentration was higher in the blood than myocardium throughout the imaging periods of 20 or 60 minutes and thus, no specific signal from the infarcted area was visible in the *in vivo* images either at 7 days or 4 weeks after coronary ligation. Blood radioactivity concentration was lower in healthy animals than after either coronary ligation or sham operation (P<0.001, Figure 3). According to radiochromatographical analysis of serial plasma samples , the amount of intact tracer rapidly decreased after injection (Figure 4). At 30 minutes after i.v.injection, 17±1.6 % of plasma total radioactivity was accounting from the intact tracer. Thus, slow blood clearance was mainly due to radiometabolites. Based on *in vivo* tracer biokinetics and stability, time point of 30 minutes post-injection was selected for *ex vivo* analyses.

Biodistribution and Myocardial Uptake of ^{68}Ga-DOTA-Peptide After MI

The results of *ex vivo* biodistribution of ^{68}Ga-DOTA-peptide are shown in Table 1 and Supplementary Fig. 1. The uptake of ^{68}Ga-DOTA-peptide in the LV myocardium was significantly higher in rats with coronary ligation than sham-operation at 7 days and 4 weeks after surgery. Blood radioactivity concentration was comparable between rats with coronary ligation or sham-operation.

The pre-treatment with MMP-2/9 inhibitor reduced tracer binding in the LV myocardium by 24 % (from 0.59 ± 0.07 SUV (n=7) to 0.45 ± 0.033 SUV (n=5), P=0.002). Excessive amount of unlabeled peptide in turn, reduced the tracer uptake in the LV myocardium by 48 % (to 0.30 ±

0.024 (n=3), P<0.001). There were also reductions in other tissues, including plasma, blood, liver, spleen, lungs and skeletal muscle (Supplementary Fig. 1).

The results of ^{68}Ga-DOTA-peptide autoradiography in LV tissue sections are shown in Figure 5 and in Table 2. Tracer uptake was homogeneous and low in the myocardium of sham-operated rats. However, there was a clear, focal increase in the uptake of ^{68}Ga-DOTA-peptide in the infarcted area both at 7 days and 4 weeks after coronary ligation. The average MI-to-remote ratio of ^{68}Ga-DOTA-peptide uptake was 2.5±0.46 (*P*=0.003) at 7 days and 3.1±0.98 (*P*=0.002) at 4 weeks. There were no differences in ^{68}Ga-DOTA-peptide uptake between remote, non-infarcted areas of rats with MI and myocardium of sham-operated either 7 days or 4 weeks after coronary ligation.

There was a strong correlation between the size of MI and the amount of ^{68}Ga-DOTA-peptide uptake in autoradiography images both in the remote and infarcted areas at 7 days after MI (Figure 6). Weaker correlations were observed at 4 weeks (Figure 6).

There was a correlation between the uptake of ^{68}Ga-DOTA-peptide in autoradiography images and areal percentage of CD68 positive macrophages in the infarcted area at 7 days after MI (Figure 7). Tracer uptake also correlated with CD31 positive area both at 7 days and 4 weeks after MI. However, there were no correlations between the uptake of ^{68}Ga-DOTA-peptide and areal percentage of collagen in the infarcted area. Tracer uptake was low in the remote, non-infarcted areas and thus, no correlations to histology were seen.

~~Zymography suggested activation of soluble gelatinases in the blood of operated rats (Supplementary Fig. 2).~~

DISCUSSION

We found that ^{68}Ga-DOTA-peptide targeting MMP-2/9 expression is taken up in the infarcted myocardium after recent ischemic injury or in the presence of an infarct scar in rat. Pre-

treatment with MMP-2/9 inhibitor or excess of unlabeled peptide significantly reduced tracer uptake indicating specific binding. In the infarcted area, tracer uptake correlated with macrophages and endothelial cells. Double staining showed co-localization of MMP-9 with CD68 positive macrophages, but both MMP-9 and MMP-2 have also been previously shown to be involved in post-ischemic neovascularization[17] after ischemic myocardial injury[7,18,19]. The tracer was rapidly metabolized, radioactivity in the blood remained high and tracer uptake in the myocardium was not detectable by *in vivo* PET.

MMP-2 and MMP-9 are potentially good targets for imaging: MMP-9 is synthesized as an inactive pro-enzyme and transported in specialized, kinesin-associated vesicles to the cell surface, where it stays associated with the cell membrane or becomes secreted[16]. To our knowledge, this is the first study to test PET tracer for imaging their cardiac expression. Although ex vivo analyses demonstrated uptake of MMP-2/9 targeting ^{68}Ga-DOTA-peptide in the infarcted myocardium, *in vivo* imaging of tracer uptake was not feasible due to low myocardium-to-blood ratio. This was most likely related to limitations in tracer properties, i.e. slow blood clearance and rapid metabolism as well as relatively low myocardial uptake. In the *in vivo* images, higher activity in the non-infarcted intervenricular septum than the infarcted area (Figure 3) as well as gradual reduction of myocardial tracer uptake is most likely related to spill over from the right or left ventricle cavity blood pool into the myocardial ROI. Spillover cannot be avoided due to limited spatial resolution of imaging and relatively high blood activity.

The reduction of tracer uptake by pre-treatment with MMP-2/9 inhibitor or unlabeled peptide indicates specific uptake in the myocardium. However, myocardial autoradiography signal could also be partly attributed to non-specific tissue uptake due to residual radiometabolites in the blood pool. A limitation of our study is that the protocol did not include direct detection of MMP-2/9 activation in the myocardium by zymography and thus, we are not able to confirm specificity

of the tracer towards the activated enzyme. Previously, the peptide has shown high selectivity towards gelatinases as compared with other MMP ~~sub~~types[12].

Autoradiography showed increased tracer uptake in the infarcted area as compared to the remote myocardium or myocardium of sham- operated rats at 1 week and 4 weeks after MI. This is consistent with localization and time course of MMP-2/9 expression after MI in previous studies[5,10,19] that have highlighted ~~isoform specific~~ differences in the regional and temporal activation patterns of MMPs after MI[5]. The importance of MMP-2/9 expression has been highlighted by the decreased rate of post-MI cardiac rupture and the attenuation of adverse LV remodeling in gene knockout animals lacking MMP-2/9 expression[8,9], as well as association to impaired regional myocardial systolic strain[5].

New tracers for imaging activation of selective MMP types are being actively developed. An example is a dual-isotope (^{177}Lu/^{125}I) radiolabeled activatable cell-penetrating peptide probe (ACPP) that is sensitive to the proteolytic activity of MMP-2/9 and subsequently becomes trapped in tissue[20]. This kind of molecular imaging probe could be an option to amplify the imaging signal intensity.

NEW KNOWLEDGE GAINED

The ^{68}Ga-labeled PET tracer targeting MMP-2/9 accumulated in the myocardium after ischemic injury, but the signal was not detectable by *in vivo* PET.

CONCLUSIONS

MMP-2/9 targeting ^{68}Ga-DOTA-peptide accumulates in the damaged rat myocardium after an ischemic injury, but tracer instability and slow clearance *in vivo* makes it unsuitable ~~inapplicable~~ for further evaluation.

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Disclosure

The authors have no conflicts of interests to report.

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Table 1. Biodistribution of ^{68}Ga-DOTA-peptide targeting MMP-2/9 expression 30 minutes after injection in rats at 7 days (7d) or 4 weeks (4w) after myocardial infarction (MI) or sham–operation as measured *ex vivo* by gamma counting of tissue samples

	7d Sham	7d MI	*P* (7d Sham vs. 7d MI)	4w Sham	4w MI	*P* (4w Sham vs. 4w MI)
Left Ventricle	0.40±0.13	0.59±0.072	0.030	0.39±0.067	0.58±0.14	0.019
Blood	1.2±0.34	1.26±0.31	1.0	1.1±0.23	1.2±0.24	0.79
Plasma	2.1±0.55	2.1±0.52	1.0	2.1±0.45	2.3±0.58	0.79
Thymus	0.20±0.049	0.23±0.059	0.39	0.20±0.025	0.23±0.085	1.0
Liver	1.4±0.19	0.75±0.32	0.071	1.5±0.33	1.2±0.58	0.38
Kidneys	3.6±2.8	3.9±1.2	1.0	3.3±0.49	3.7±1.80	0.86
Spleen	1.4±0.25	0.97±0.48	0.267	1.3±0.21	1.1±0.54	0.86
Lungs	1.0±0.021	0.81±0.26	0.143	1.4±0.10	1.9±0.58	1.0
Skeletal Muscle	0.15±0.045	0.17±0.020	0.432	0.13±0.020	0.17±0.11	0.54
Urine	230±130	270±160	0.88	190±180	120±37	0.004

The results are shown as standardized uptake value (SUV, mean±SD with two significant figures).

Table 2. Uptake of ^{68}Ga-DOTA-peptide in the infarcted area (MI) and remote non-infarcted area of the rat myocardium at 7 days (7d) or 4 weeks (4w) after coronary ligation or sham-operation as determined by autoradiography of tissue cryosections

	7d Sham	7d MI	4w Sham	4w MI	*P* (7d Sham vs. 7d MI)	*P* (4w Sham vs. 4w MI)
MI	ND	29±2.8[a]	ND	31±5.0[c]	0.003	0.002
Remote	12±3.4	12±3.0[b]	11±2.4	11±4.6[d]	0.88	0.72

The results are expressed as photostimulated luminescence/mm^2 (PSL/mm^2, mean±SD). [a]=p=0.003 vs. Remote, [b]=p=0.88 vs. Sham, [c]=p=0.002 vs. Remote, [d]=p=0.72 vs. Sham *ND, not detected.*

FIGURE LEGENDS

Figure 1. Structure of ^{68}Ga-DOTA-peptide tracer (Cys$^{3-10}$; H-Gly-Ala-Cys-Leu-Arg-Ser-Gly-Arg-Gly-Cys-Gly-PEG(3)-DOTA-^{68}Ga).

Figure 2. Histology and immunohistochemistry of rat myocardium after coronary ligation or sham-operation. Micrographs show Masson's trichrome stained left ventricular myocardium of a sham-operated animal (**a**) and infarcted area at 7 days (**b**) and 4 weeks (**c**) after coronary ligation. Myocytes appear red and collagen fibers blue. Micrographs of tissue sections from the infarcted area 7 days after ligation stained with immunohistochemistry show MMP-2 positive cells in remote (**d**) and infarcted area (**e**), MMP-9 positive cells in remote (**f**) and infarcted area (**g**), CD31 positive endothelial cells (**h**), and CD68 positive macrophages (**i**),in brown color. Double staining shows MMP-9 positivity in brown color and either α smooth muscle actin (α-SMA) in fibroblasts or smooth muscle cells (**j**) or CD68 staining of macrophages as blue color (**k**). Note that MMP-9 co-localizes with many CD68 positive cells (arrows). In contrast, MMP-9 does not co-localize with α-SMA positive cells. Scale bars are 50 μm.

Figure 3. *In vivo* biokinetics of ^{68}Ga-DOTA-peptide in rats. Time-activity curves of sham-operated rats (**a**, n=5), blood of healthy, non-operated animals (**b**, n=3), or rats with myocardial infarction (MI) either 7 days (**c**, n=3) or 4 weeks (**c**, n=7 for 20 min and n=3 for 60 min PET imaging) after coronary ligation. Transaxial PET/CT images show tracer uptake in different time points (CT reference **d** , 0-2 min **e**, 2-10 min **f**, 10-60 min **g**) 4 weeks after myocardial infarction.

Figure 4. *In vivo* stability of intravenously administered ^{68}Ga-DOTA-peptide in rat plasma at 4 weeks after coronary ligation (**a**). At 30 min post-injection, two radioactive metabolites were detected in plasma with radio-HPLC analysis (**b**).

Figure 5. Distribution of^{68}Ga-DOTA-peptide in rat myocardium as detected by autoradiography. Micrographs of cross sections of the left ventricle stained with Masson's trichrome after sham-

operation (**a**) and either 7 days (**c**) or 4 weeks (**e**) after coronary ligation. Autoradiographs of the same sections show homogeneous, low uptake of ^{68}Ga-DOTA-peptide in the myocardium of sham-operated rat (**b**) and in the remote, non-infarcted myocardium of rats with coronary ligation (**d** and **f**). Note that there is focally increased uptake of ^{68}Ga-DOTA-peptide co-localizing with the infarcted areas (arrows in **c**-**f**) both at 7 days (**d**) and 4 weeks (**f**) after MI. Scale bar is 5 mm.

Figure 6. Correlations between ^{68}Ga-DOTA-peptide uptake in autoradiography and the size of myocardial infarction (MI) at 7 days (**a** and **b**) after coronary ligation or 4 weeks (**c** and **d**) after coronary ligation in the remote (**a** and **c**) and infarcted (**b** and **d**) areas. r_s, Spearman's correlation coefficient; *PSL*, photostimulated luminescence; *circ-%*, percentage of infarcted LV circumference.

Figure 7. Scatter plots show correlations between ^{68}Ga-DOTA-peptide uptake and the areal percentages of CD68 positive macrophages in infarcted area at 7 days (**a**) or 4 weeks (**b**) as well as CD31 staining of endothelial cells in infarcted area at 7 days (**c**) or 4 weeks (**d**) after coronary ligation. r_s, Spearman's correlation coefficient; *PSL*, photostimulated luminescence.