Ultrasound Inflammation Imaging in Rats with Myocardial Ischemia-Reperfusion: Evaluation by Non-Specific Targeted Contrast Microbubbles

Toshihiko Asanuma*, Ryu Mabuchi*, Kasumi Masuda*, Hatsue Ueda-Ishibashi†, Satoshi Nakatani*

* Department of Health Sciences, Division of Functional Diagnostics, Osaka University Graduate School of Medicine, Suita, Osaka, Japan

† Department of Pathology, National Cardiovascular Center, Suita, Osaka, Japan

Reprint request and correspondence:

Dr. Satoshi Nakatani

Department of Health Sciences, Division of Functional Diagnostics, Osaka University Graduate School of Medicine, 1-7 Yamadaoka, Suita, Osaka, 565-0871, Japan

E-mail: nakatani@sahs.med.osaka-u.ac.jp

Abstract

Background: Reports on ultrasound inflammation imaging with non-specific targeted microbubbles in the heart have been scarce. We investigated whether inflammation induced by myocardial ischemia-reperfusion in rats could be evaluated by ultrasound inflammation imaging with non-specific targeted microbubbles.

Methods: Six rats subjected to 30 min of occlusion of the left anterior descending artery (LAD) followed by 4 hours of reperfusion (ischemia group) and 4 rats subjected to the sham operation (sham group) were used. Ultrasound contrast imaging was performed twice 4 hours after reperfusion and non-circulating signal intensity (SI), which reflects the signal derived from microbubbles phagocytosed by neutrophils in inflamed tissue, was calculated by the SI difference between the initial and subsequent imaging both in the LAD and non-LAD areas. The accumulation of neutrophils was confirmed by myeloperoxidase (MPO) staining.

Results: Non-circulating SI in the LAD area was significantly greater for the ischemia group than the sham group (5.19 ± 2.19 [ischemia] vs. 0.31 ± 0.13 [sham] dB, $p < 0.01$). Non-circulating SI in the LAD area was significantly higher than that in the non-LAD area when compared in the same rat of the ischemia group (5.19 ± 2.19 [LAD] vs. 0.18 ± 0.64 [non-LAD] dB, $p < 0.01$). MPO positive cells were confirmed in the LAD area of the ischemia group.

Conclusion: Inflammation induced by myocardial ischemia-reperfusion in rats could be quantitatively assessed by ultrasound inflammation imaging with non-specific targeted microbubbles.

Keywords: contrast, echocardiography, inflammation, microbubble

Contrast ultrasonography has been evolved as an imaging technique to allow ultrasound visualization of tissue perfusion dynamics by the injection of contrast microbubbles into vessels. Moreover, ultrasound molecular imaging has recently possible with the development of novel site-targeted microbubbles. For molecular imaging, microbubbles can be targeted either by intrinsic properties of the shell constituents that interact with upregulated cell receptors (non-specific targeted microbubbles), or by surface conjugation of specific ligands or antibodies that bind to disease-related markers (specific targeted microbubbles). Past studies have clarified that non-specific targeted microbubbles with shells composed of albumin or lipid can bind to activated neutrophils and monocytes retained within the microcirculation of inflamed tissue. Microbubbles attached to the surface of neutrophils and monocytes are phagocytosed and still react acoustically to ultrasound [1, 2]. Since a strong correlation exists between severity of inflammation and ultrasound signals of microbubbles at inflamed sites, the location and severity of inflammation can be imaged with contrast ultrasonography [3].

Inflammation provoked by myocardial ischemia-reperfusion is expected to be assessed by this technique. However, reports on ultrasound inflammation imaging in the heart have been scarce. We therefore investigated whether inflammation induced by myocardial ischemia-reperfusion in rats could be evaluated by ultrasound inflammation imaging with non-specific targeted microbubbles.

Methods

Animal preparation

Six male Sprague-Dawley rats subjected to myocardial ischemia-reperfusion (ischemia group) and 4 rats subjected to sham operation (sham group) were used in this study. The rats were anesthetized by administering sodium pentobarbital (50 μg/g) intraperitoneally, and a polyethylene catheter was inserted into the left femoral vein to administer anesthetic and contrast microbubbles. Anesthesia was maintained by intravenously administering pentobarbital as necessary. After tracheal intubation, the intubation tube was connected to the respirator, and artificial ventilation was performed. An intercostal incision was then placed to open the chest and pericardiotomy was performed. The proximal portion of the left anterior descending

artery (LAD) was occluded for 30 min with 6-0 surgical suture, followed by reperfusion for 4 hours to cause myocardial inflammation based on ischemia-reperfusion injury. In the sham group, after pericardiotomy, the rats were left alone for 30 min to match the duration of ischemia and for 4 more hours to match the duration of reperfusion.

Image acquisition and data analysis

Ultrasound image acquisition was performed with an Aplio ultrasound system (Toshiba, Otawara, Japan). At 15 min after LAD ligation, the ischemic LAD area was evaluated by conventional myocardial contrast echocardiography (MCE). Transmitting and receiving frequencies were 4.5 and 9.0 MHz, respectively, and mechanical index was set at 1.0. Definity (Bristol-Myers Squibb Medical Imaging, Billerica, Massachusetts), which is non-specific targeted contrast microbubbles, was diluted 1:30 in normal saline and administered intravenously by 0.1 mL of bolus injection. Intermittent images of the end-diastolic left ventricular short-axis were acquired at every 30th cardiac cycle. MCE was repeated after reperfusion to confirm success of reperfusion.

Ultrasound inflammation imaging was performed according to the method of Lindner et al [3]. After 4 hours of reperfusion, 0.1 mL of Definity diluted 30-fold was again injected as a bolus. Ultrasound insonation for imaging was not resumed until 12 min after injection to wait for neutrophils to phagocytose microbubbles, and then intermittent images were acquired at every 2nd cardiac cycle (short pulsing interval). The initial frame of these images was used to assess the signal from both retained and freely circulating microbubbles. After microbubbles were destroyed by subsequent pulses, the interval of intermittent images was changed into every 30th cardiac cycle (long pulsing interval). Ten frames of these images were used to assess the signal from freely circulating microbubbles (Fig. 1).

Inflammation images were analyzed using Image Lab software (Toshiba, Otawara, Japan). In the ischemia group, circular regions of interest were set at the center of the LAD area detected by conventional MCE and at the opposite non-LAD area for the analysis of signal intensity (SI). In the sham group, a region of interest was set in the area supposed to be perfused by the LAD. SI on the initial frame of short pulsing interval was subtracted by averaged SI on 10 frames of long pulsing interval as non-circulating SI. This

non-circulating SI theoretically reflects the signal derived from microbubbles phagocytosed by neutrophils [3].

Myeloperoxidase (MPO) staining

For both ischemia and sham groups, the heart was excised immediately after obtaining echocardiographic data, fixed in 20% formalin solution, and sliced into 5-mm sections parallel to the left ventricular short-axis plane. MPO staining was then performed to observe the accumulation of neutrophils and monocytes as an indicator of inflammation, and MPO-positive cells in myocardium were observed under microscopy.

Statistical analysis

Data were expressed as mean ± SD. The comparison of non-circulating SIs in the LAD area between the ischemia and sham groups was performed by the unpaired *t* test. The comparison of non-circulating SIs in the ischemia group between the LAD and non-LAD areas was performed by the paired *t* test. Values of $p < 0.05$ were considered statistically significant.

Results

There were no rats excluded from the data analysis due to poor quality of imaging. In conventional MCE, clear contrast defect was demonstrated during the LAD ligation for the ischemia group, and successful reperfusion was confirmed in all the cases (Fig. 2).

By using ultrasound inflammation imaging, myocardial contrast enhancement was shown in the LAD area and less contrast was observed in the non-LAD area on the initial frame of short pulsing interval for the ischemia group. In contrast, contrast enhancement was hardly shown in both areas on the frame of long pulsing interval (Fig 3). Myocardial contrast enhancement was not observed in the supposed LAD area on the initial flame of short pulsing interval for the sham group.

Compared to the sham group, non-circulating SI in the LAD area was significantly greater for the ischemia group (Fig 4). Non-circulating SI in the LAD area was significantly higher than that in the non-LAD area when compared in the same rats of the ischemic group (Fig 4).

MPO staining confirmed neutrophil and monocyte accumulation in the LAD area for the ischemia group. However, no accumulation of the MPO positive cell was seen in the LAD area for the sham group or in the non-LAD area for the ischemia group (Fig 5).

Discussion

Lindner et al. reported that ultrasound inflammation imaging by contrast microbubbles with albumin or lipid shells, which are phagocytosed by activated neutrophils and monocytes, is possible for the kidney [3]. Although this non-specific targeting method can be used for inflammation imaging in several organs, there are only a few reports with regard to the application of this method in the heart [4, 5]. Based on this method, the present study investigated whether inflammation in the heart, a dynamic organ, could be visualized in rats with myocardial ischemia-reperfusion. The results showed that non-circulating SI in the LAD area was significantly higher for the ischemia group than for the sham group, suggesting that visualization of inflammation associated with ischemia-reperfusion in rats is possible using non-specific targeted microbubbles. Furthermore, non-circulating SI was significantly higher in the LAD area than in the non-LAD area for the ischemia group, and neutrophil accumulation by MPO staining was confirmed in ischemic regions, suggesting that the location of inflammation can be evaluated using this method.

In ultrasound inflammation imaging, non-circulating SI theoretically reflects the signal derived from retained (non-circulating) microbubbles. The cause of increased non-circulating SI in the ischemic area seems to be due to the microbubbles attached to or phagocytosed by neutrophils [3]. However, non-specific targeted microbubbles might be retained within the microcirculation of inflamed tissue by other mechanisms such as low flow after reperfusion or direct interaction with damaged endothelial cells.

In this study, ultrasound inflammation imaging was not resumed until 12 min after injection of microbubbles according to Lindner’s method [3]. Consequently, myocardial contrast enhancement in the LAD area was shown on the initial frame of short pulsing interval and was hardly shown on the frame of long pulsing interval. Therefore, the 12 min of waiting time seems to have been reasonable. At the shorter waiting time, the concentration of freely circulating microbubbles in the blood pool would be high. This

situation is undesirable for detecting retained microbubbles. At the longer waiting time, however, ultrasound signal derived from phagocytosed microbubbles might be weak.

Neutrophil avidity for lipid microbubbles is greatly enhanced by incorporating phosphatidylserine into the shell, which increases microbubble retention in inflamed tissue [3]. Christiansen et al. reported that the severity and extent of postischemic myocardial inflammation could be evaluated with microbubbles containing phosphatidylserine in dogs [4]. The shell of microbubbles we used does not contain phosphatidylserine. The use of other microbubbles containing phosphatidylserine might be more effective.

The non-specific targeting method is most effective when used to image acute inflammation in which the density of activated neutrophil on the endothelial cell surface is high. The assessment of inflammation in the ischemia-reperfusion condition would be therefore suitable for this technique. In contrast, this technique would not be suitable for the detection of sub-acute or chronic inflammation because activated neutrophils undergo transendothelial migration and their density on the endothelial cell surface is no longer high. For the assessment of this inflammation condition, it is reported that specific targeted microbubbles, which bear antibodies or other ligands to endothelial cell adhesion molecules (i.e., selectins, intercellular adhesion molecule-1, and vascular cell adhesion molecules-1), are useful [6-9].

Clinical implications

Neutrophils accumulation, which is observed in the myocardium damaged by ischemia-reperfusion, contributes to myocellular injury and microvascular no-reflow. Because conventional echocardiography cannot assess such molecular information, development of ultrasound inflammation imaging has been desired in clinical settings. If the non-specific targeting method used in this study is feasible in patients with acute coronary syndrome, it may be valuable not only for assessing the severity of ischemia-reperfusion injury but also for predicting their prognosis. Moreover, because ultrasound inflammation imaging allows after-the-fact recognition of ischemic insult, it might be able to be used for myocardial ischemic memory imaging in patients with a history of chest pain [10].

Study limitations

We did not assess interobserver and intraobserver variability of non-circulating SI. Although SDs

of non-circulating SI in our results were not large, reproducibility of ultrasound inflammation imaging should be addressed in further studies.

Unfortunately, we did not test the relationship between non-circulating SI and the degree of neutrophil and monocyte accumulation. In Christiansen's result, the non-circulating SI correlated with tissue MPO activity [4] However, it should be further studied whether microbubbles without phosphatidylserine can produce the similar result.

Conclusion

In this study, we found that inflammation induced by myocardial ischemia-reperfusion in rats could be quantitatively assessed by ultrasound inflammation imaging with non-specific targeted microbubbles. This technique may allow ultrasound visualization of acute myocardial inflammation in the clinical settings.

References

1. Lindner JR, Dayton PA, Coggins MP, Ley K, Song J, Ferrara K, et al. Noninvasive imaging of inflammation by ultrasound detection of phagocytosed microbubbles. Circulation 2000;102:531-8
2. Lindner JR, Coggins MP, Kaul S, Klibanov AL, Brandenburger GH, Ley K. Microbubble persistence in the microcirculation during ischemia/reperfusion and inflammation is caused by integrin- and complement-mediated adherence to activated leukocytes. Circulation 2000;101:668-75
3. Lindner JR, Song J, Xu F, Klibanov AL, Singbartl K, Ley K, et al. Noninvasive ultrasound imaging of inflammation using microbubbles targeted to activated leukocytes. Circulation 2000;102:2745-50
4. Christiansen JP, Leong-Poi H, Klibanov AL, Kaul S, Lindner JR. Noninvasive imaging of myocardial reperfusion injury using leukocyte-targeted contrast echocardiography. Circulation 2002;105:1764-7
5. Kondo I, Ohmori K, Oshita A, Takeuchi H, Yoshida J, Shinomiya K, et al. Leukocyte-targeted myocardial contrast echocardiography can assess the degree of acute allograft rejection in a rat cardiac transplantation model. Circulation 2004;109:1056-61
6. Villanueva FS, Jankowski RJ, Klibanov S, Pina ML, Alber SM, Watkins SC, et al. Microbubbles targeted to intercellular adhesion molecule-1 bind to activated coronary artery endothelial cells. Circulation 1998;98:1-5.
7. Lindner JR, Song J, Christiansen J, Klibanov AL, Xu F, Ley K. Ultrasound assessment of inflammation and renal tissue injury with microbubbles targeted to P-selectin. Circulation 2001;104:2107-12
8. Hamilton AJ, Huang SL, Warnick D, Rabbat M, Kane B, Nagaraj A, et al. Intravascular ultrasound molecular imaging of atheroma components in vivo. J Am Coll Cardiol 2004;43:453-60
9. Kaufmann BA, Lewis C, Xie A, Mirza-Mohd A, Lindner JR. Detection of recent myocardial ischaemia by molecular imaging of P-selectin with targeted contrast echocardiography. Eur Heart J 2007;28:2011-7
10. Villanueva FS, Lu E, Bowry S, Kilic S, Tom E, Wang J, et al. Myocardial ischemic memory imaging with molecular echocardiography. Circulation 2007;115:345-52

Figure Legends

Fig. 1 Protocol of ultrasound inflammation imaging (according to the method of Lindner et al. [3]). Ultrasound insonation was stopped until 12 min after microbubble injection to wait for neutrophils to phagocytose microbubbles, and then intermittent images were acquired at short pulsing interval (PI). The initial frame of these images was used to assess the signal from both retained and freely circulating microbubbles. After microbubbles were destroyed by subsequent pulses, long PI images were acquired to assess the signal from freely circulating microbubbles.

Fig 2 Myocardial contrast echocardiography for the assessment of the area perfused by the left anterior descending artery (LAD). Contrast defect (arrow heads) was clearly demonstrated during the LAD ligation (left) and contrast filling in the defect area was confirmed after reperfusion (right).

Fig 3 Ultrasound inflammation imaging using non-specific targeted microbubbles. After 4 hours of reperfusion, contrast enhancement (arrow heads) was shown in the left anterior descending artery (LAD) area and less contrast was observed in the non-LAD area on the initial frame of short pulsing interval, which was used to assess the signal from both retained and freely circulating microbubbles (left). Contrast enhancement was hardly shown in both areas on the frame of long pulsing interval, which was used to assess the signal from freely circulating microbubbles (right).

Fig 4 Non-circulating signal intensity (SI) derived from ultrasound inflammation imaging. This index reflects the signal derived from microbubbles phagocytosed by neutrophils in inflamed tissue. Non-circulating SI in the left anterior descending artery (LAD) area was significantly greater for the ischemia group than the sham group (upper). Non-circulating SI in the LAD area was significantly higher than that in the non-LAD area when compared in the same rat of the ischemia group (lower). *$p < 0.01$ vs. sham, †$p < 0.01$ vs. non-LAD.

Fig 5 Myeloperoxidase (MPO) staining for the assessment of neutrophil and monocyte accumulation. MPO positive cells were confirmed in the left anterior descending artery (LAD) area for the ischemia group. However, MPO positive cells were not seen in the LAD area for the sham group or in the non-LAD area for the ischemia group.