Lymphology 45 (2012) 80-86

RECONSTITUTION OF MYOCARDIAL LYMPHATIC VESSELS AFTER ACUTE INFARCTION OF RAT HEART

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ABSTRACT

We investigated the regeneration of cardiac lymphatic vessels and capillaries in the infarcted myocardiac zones after acute myocardial infarction in rats. The anterior descending artery of the heart was ligated for infarction and both immunohistochemistry and immunofluorescence were used to detect pathological changes of lymphatic vessels in infarcted zone (IZ), infarcted margin zone (MZ) and remote margin zone (RMZ) on days 7, 14, 21, and 28 after surgery. Dynamic variation of lymphatic vessels existed in IZ, MZ and RMZ at different stages after surgery. At day 7, lymphatic vessels and capillaries were not seen in the IZ, very thin lymphatic capillaries were obviously increased in the inner layer of the margin zone, and enlarged and increased lymphatic capillaries were found in the outer layer of margin zone. At 14 days, a few sparsely arranged lymphatic capillaries were observed in the IZ without marked changes in the MZ. At 21 days, constricted regenerating lymphatic capillaries in MZ were decreased, and lymphatic vessels exhibited sprouting towards the IZ. At 28 days, more lymphatic capillaries emerged in the IZ, and the morphology and number of lymphatic vessels and capillaries had returned to normal. There were no marked changes of lymphatic vessels and capillaries in RMZ compared to control myocardium at the 4 time points. This study demonstrates varied remodeling of lymphatic vessels and capillaries in the IZ and MZ after acute myocardial infarction, and these changes in lymphatic vessels likely play an important role for recovery of infarcted myocardiac function.

Keywords: cardiac lymphatics, lymphatic capillary, lymphatic vessel, myocardiac infarction, infarction recovery, myocardial fibrosis, rat heart

Myocardial ischemia not only injures myocardial cells, but it also affects the morphology and functions of lymphatic vessels (both large diameter vessels and small capillaries) in the myocardium. Cardiac blood vessels and lymphatic vessels play different roles during the course of myocardial infarction. The former mainly increase and improve myocardial nutrition before the formation of granulation scar, while the latter mainly drain protein and tissue fluid during fibrosis and scar formation. Several previous studies have shown that disordered lymphatic drainage may cause cardiac pathological changes such as myocardial interstitial fibrosis (1-4), conduction disturbances (5,6), acute and chronic coronary artery injury (7-10), reduced myocyte contraction, and others (11). It is obvious that these changes in lymphatic vessels cannot be ignored in the occurrence and development of myocardial ischemia. Although some previous studies of lymphatic vessel injury in the myocardium

have been conducted, studies focusing on morphological changes in myocardial lymphatic vessels after myocardial ischemia have rarely been examined. Furthermore, the existing studies concerning cardiac lymphatic vessels in ischemic heart disease have not been translated from basic research into clinical applications. In order to provide related morphological evidence for studying the development of ischemic heart disease and prognosis, this study investigated changes in cardiac lymphatic vessels following acute myocardial infarction using a rat model of cardiac ischemia.

MATERIALS AND METHODS

Animals

Twenty-five healthy adult Sprague Dawley rats (male or female, average 2.3 weeks old and 300 ± 4.3 g) were divided randomly into five groups: normal control, and 4 experimental groups. The experimental groups were subdivided into 7, 14, 21, and 28 day groups according to experimental time intervals. All rats were obtained from Xinxiang Medical College animal center, and the study was approved by the Committee of Animal Administration of the Xinxiang Medical University.

Model of Myocardial Infarction

Rats were placed under deep anesthesia with 10% chloral hydrate (0.3ml/100g body weight), secured on the operating table for small animals and intubated. Animals were then attached to a small animal ventilator (model 6025/7025, RWD Life Science Limited, Shenzhen, China). A normal electrocardiogram was traced as control. Then the chest cavity was opened, the heart exposed, pericardium opened, and the anterior descending artery of left coronary artery was ligated with 6-0 suture. The myocardial blood supply zone of anterior descending artery could be observed to darken, and the two-lead ECG indicated ST-segment elevation greater than 0.1mv. The ECG elevation continued for more than 10 minutes demonstrating a successful procedure. The ECG was traced for 15-20 min. The chest cavity was closed if heart rhythm and voltage were normal. Control group was carried out using the same procedure without ligating the descending artery branch.

Sample Preparation and Sectioning

The chest cavity was opened again at day 7, 14, 21, or 28 as appropriate for each group. The fresh heart was immediately removed from the pericardium and placed into PBS. The ventricles were opened along with cardiac left and right lateral margins, and large arteries and excess tissue connecting the cardiac base were quickly removed. A tissue block near the cardiac apex was cut transversely at a point 2mm from the inferior border of ligation line. This block included tissue from the infarction zone, infarction marginal zone, and remote infarction zone. Processed tissue was cut into 5 μm sections in the cryostat and stained by H&E, Masson, and immunochemistry techniques.

Immunohistochemistry Staining

Sections were pre-fixed with 4°C cold acetone for 30 minutes, treated with H_2O_2 in PBS for 15 min to inhibit intrinsic peroxidase activity, and blocked for 20 minutes to prevent non-specific antibody binding. They were then incubated overnight at 4°C with rabbit anti-rat podoplanin polyclonal antibody (1:100, Sigma). After rinsing in PBS, slides were incubated for 30 minutes at 37°C with poly HRP anti-rabbit IgG (PV-9003, Beijing Zhongshan). Slides were rinsed again with PBS and stained with DAB, counter-stained in hematoxylin, dehydrated, and mounted. Control immunostaining was carried out using the same procedure with PBS replacing the primary antibody.

Immunofluorescence Staining

Slides were rinsed with PBS after antigen retrieval by sodium citrate solution. Nonspecific antibody binding was prevented using 5% BSA blocking solution at 37°C for 30 minutes. They were then incubated overnight at 4°C with rabbit anti-rat podoplanin polyclonal antibody (1:100). After rinsing in PBS, slides were incubated for 1hr at 37°C with Cy3 anti-rabbit IgG (Pik-day Institute of Biotechnology), rinsed again in PBS, and mounted with anti-fluorescent quencher solution. Finally, slides were examined and photographed using a laser confocal microscope.

RESULTS

Pathologic Changes of Myocardial Infarction

H&E and Masson staining demonstrated that after coronary artery ligation, the region can be divided into the infarcted zone, infarcted margin zone, and the remote zone. The cardiac wall in myocardial infarction became thinner, and it contained large amounts of fiber cells, fibroblasts, and collagen fibers. Myocardial cells disappeared in most infarcted areas with only occasional scattered islands of myocardial cells seen and large amounts of inflammatory cells infiltrating through the margin of infarcted area. Thus, the model was successful (*Fig. 1*). At days 7-28 after surgery, the infarct size gradually decreased and was slowly replaced by scar tissue. In addition, myocardial cells and lymphatic capillaries gradually grew from the margin zone to the infarcted zone.

Expression of Normal Myocardial Lymphatic Vessels

Normal myocardial tissue contained both lymphatic vessels and capillaries. The lumens of lymphatic vessels were irregular with thin walls. On longitudinal sections, they arranged parallel to myocardial cells, and their appearance was irregular and cord-like. The lumen alternated between large and small. Occasionally, a few horizontal cross-cut lymphatic vessels were seen (*Fig. 1*). A large and dense distribution of lymphatic vessels and small capillaries was seen in cross-sections of the cardiac muscle, the former with a larger lumen, and the latter with a smaller lumen. Immunofluorescence with laser confocal microscopy clearly demonstrated lymphatic vessels in normal myocardial tissue.

Marker Expression of Lymphatic Vessels in Myocardial Region

The shape and number of lymphatic vessels in the myocardial infarcted region was significantly different from normal myocardial tissue by microscopy. The overall variation in pattern demonstrated a reduction or elimination of lymphatic vessels in the infarcted zone, and the infarcted margin zone was divided into the inner layer (close to the site of the infarcted zone) and the outer layer (outside the site of infarcted zone). Over the time course of the experiment, the number of lymphatic vessels gradually increased. The inner lymphatic vessels were arranged densely with smaller diameters, the outer lymphatic vessels were sparser and enlarged significantly, and the number of lymphatic vessels in the outside area was slightly less compared to the margin zone, but increased compared to the infarcted zone (*Figs. 2-5*). The immunofluorescence experiments with confocal microscopy detection showed the same results as immunohistochemistry.

Over the 28 day time period, different dynamic changes of the three zones were seen. At 7 days, the infarcted zone became larger, and no lymphatic vessels or capillaries were observed in the infarcted zone. The lymphatic vessels in the margin zone were clearly arranged in inner and outer layers. More small lymphatic capillaries appeared in the inner zone while larger lymphatic capillaries were seen in the outer zone (*Fig. 2*).

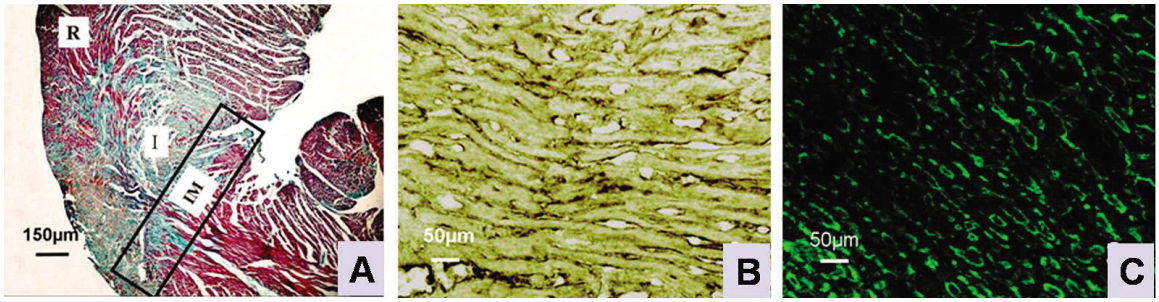

Fig. 1. A) Heart section with Masson staining demonstrates the different zones of infarcted tissues. The ventricular wall displayed attenuation and scarring (blue area) after ligation the anterior descending branch. I=infarcted zone, IM=infarcted margin zone, R=remote infarcted zone. B) Immunohistochemical staining for podoplanin demonstrates lymphatic vessels and capillaries in the normal myocardium. C) Immunofluorescence staining for podoplanin demonstrates lymphatic vessels and capillaries in the normal myocardium.

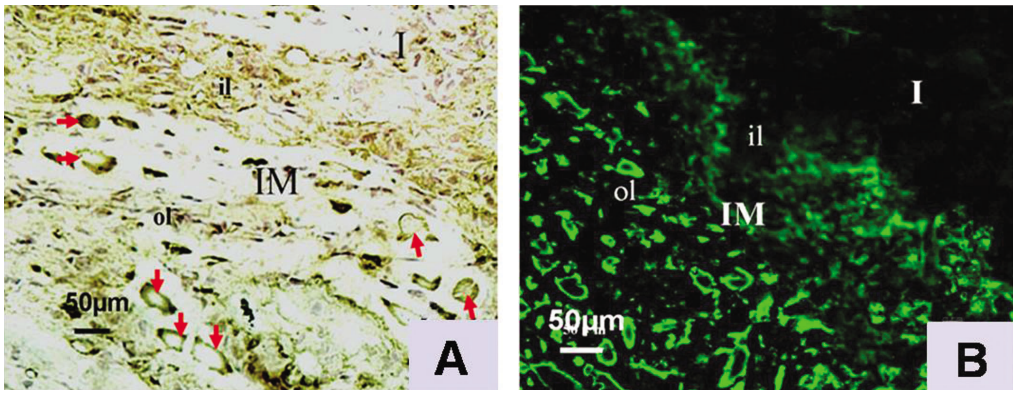

Fig. 2. Changes in lymphatic vessels and lymphatic capillaries in the infarcted zone and infarcted margin zone at 7 days after surgery demonstrated by immunohistochemistry with hematoxylin counter stain (A) and by immunofluorescence (B) for podoplanin. In A, arrows show enlarged lymphatic vessels.

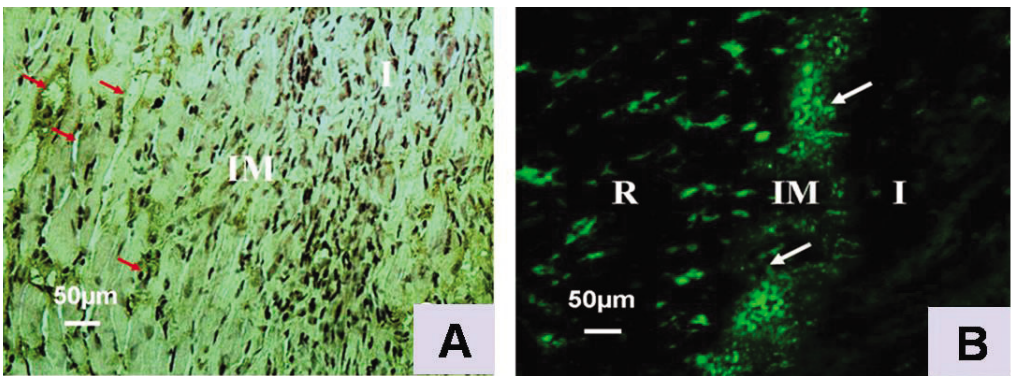

Fig. 3. Changes in lymphatic vessels and lymphatic capillaries in the infarcted zone and infarcted margin zone at 14 days after surgery demonstrated by immunohistochemistry with hematoxylin counter stain (A) and by immunofluorescence (B) for podoplanin. In A, arrows show enlarged lymphatic vessels. In B, arrows shows many small capillaries in the inner layer of margin zone.

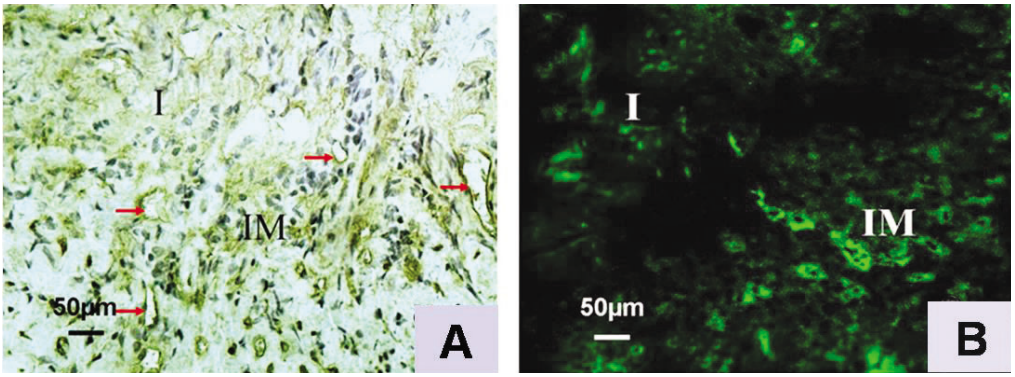

Fig. 4. Changes in lymphatic vessels and lymphatic capillaries in the infarcted zone and infarcted margin zone at 21 days after surgery demonstrated by immunohistochemistry with hematoxylin counter stain (A) and by immunofluorescence (B) for podoplanin. In A, arrows show enlarged lymphatic vessels. In B, a few lymphatic vessels and capillaries can be seen emerging in the infarcted zone.

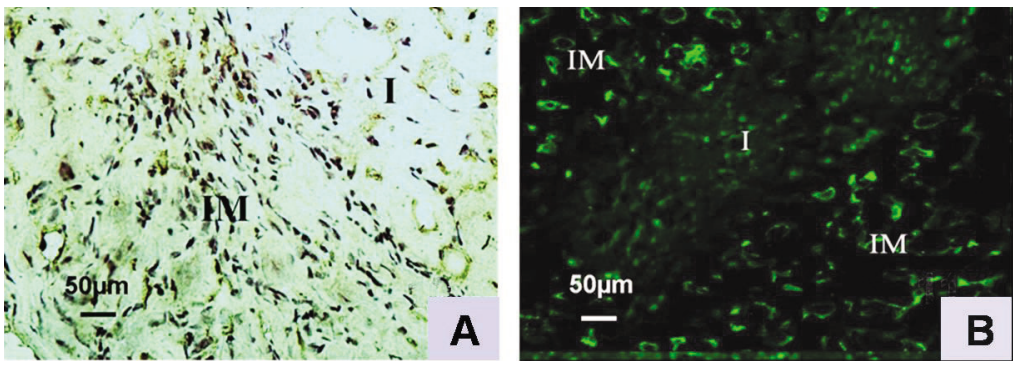

Fig. 5. Changes in lymphatic vessels and lymphatic capillaries in the infarcted zone and infarcted margin zone at 28 days after surgery demonstrated by immunohistochemistry with hematoxylin counter stain (A) and by immunofluorescence (B) for podoplanin. In B, more lymphatic vessels and capillaries can be seen emerging in the infarcted zone.

At 14 days, a few sparse lymphatic capillaries in the infarcted zone could be seen, and the edge of the inner layer contained a dense capillary layer of small lymphatic capillaries. The number of lymphatic vessels in the outer layer of the margin zone was reduced compared to 7 days (*Fig. 3*). After 21 days, scattered lymphatic vessels in the infarcted zone were seen, and the inner and outer layers of the margin zone were not obvious. Small and concentrated lymphatic capillaries were reduced in the infarcted margin zone, and the number of thickened lymphatic vessels was reduced and the diameter decreased. On the edge of the margin zone, a few bud-like new lymphatic capillaries sprouted into the infarct zone (*Fig. 4*). At 28 days, more scattered lymphatic capillaries in the infarcted zone were observed, and the number of lymphatic vessels and capillaries was decreased in the margin zone. However, the diameter of the lymphatic vessels was not significantly different compared to those in the remote infarcted zone (*Fig. 5*). At 7 to 28 days, the remote zone lymphatic vessels and capillaries displayed no significant changes compared to normal control cardiac tissue.

DISCUSSION

Myocardial infarction is caused by a dysfunction of coronary blood flow leading to myocardial damage and changes in cardiac function. Therefore, clinical therapy is often arrived at restoring infarct-related artery revascularization to improve myocardial function. Less recognized is that at the same time cardiac blood vessel abnormalities and myocardial damage occur, myocardial lymphatic vessels and capillaries are also damaged. Unfortunately, this relationship has not attracted much scientific interest. The cardiac lymph circulation and the myocardial lymphatic vessels are important components of the physiology and pathology of the heart, but the literature on myocardial lymphatic vessels is not robust. At present, most cardiac lymphatic research focuses on blocking the cardiac lymphatic circulation, and then observing cardiac structural and functional changes. These studies have shown that interruptions in cardiac lymph flow can lead to changes of cardiac structure, abnormal electrocardiogram, decline of cardiac output, increased central venous pressure, prolonging of cycle time, and action potential changes (2-9). However, studies concerning changes in the lymphatic vessels themselves in heart disease and cardiovascular disease are surprisingly rare (10,12,13). Therefore, further study of the changes in morphology and function of lymphatic vessels after myocardial infarction may guide future clinical investigation and treatment for myocardial infarction.

This study indicated that following myocardial infarction in rats, the infarcted margin and remote margin zones exhibited changes in the shape and number of lymphatic vessels. As time post-infarction extended, lymphatic vessels in the zones regenerated continuously and simultaneously. After 7 days, lymphatic vessel number in the infarcted zone was minimal or non-existent. At 14 days, sparse lymphatic capillaries appeared in small infarcted regions, and denser bud-like new lymphatic capillaries emerged from the margin zone into the infarcted zone. By 28 days, major lymphatic capillaries were seen in the infarcted zone. These results indicated that following myocardial infarction, the myocardium underwent necrosis where the lymphatic vessels greatly diminished or disappeared. As restoration of myocardial infarction progressed, lymphatic capillaries in the infarcted zone grew continuously. At 14 days, small, dense lymphatic capillaries seen around the infarcted margin zone formed an inner layer suggesting that the lymphatic vessels of the infarcted margin zone sprouted and/or reconstituted to converge from the periphery into the infarcted central area. This type of growth has previously been demonstrated in other tissues (14). After myocardial infarction, the largest change in lymphatic vessels was found in the margin zone. In the days following infarction, the number of small lymphatic vessels near the infarcted tissue gradually declined and the lumens gradually enlarged. At the same time, other lymphatic vessels started to emerge from the margin zone into the infarcted tissue. While the number of lymphatic vessels in the remote zone did not significantly change, the lumens slightly enlarged, and by 14 days this small change had returned to normal.

In the early stage post-infarction, the margin zone was in acute aseptic inflammation, and the metabolites in the infarcted zone and margin zone (i.e., macromolecules and interstitial fluid) dramatically increased. This phenomenon coupled with additional inflammatory factor stimulation likely also caused the lymphatic vessels to increase and enlarge to meet the needs of myocardial metabolism. In fact, this could actually be considered lymphatic compensation for altered cardiac function (15), and this compensation plays an essential role in improving cardiac microenvironment by reducing cardiac pressure and decreasing interstitial edema (16). Stolyarov and his colleagues

demonstrated that lymphatic drainage in a rat model of myocardial infarction plays an important role in handling the myocardial interstitial fluid during myocardial ischemia (12). If the lymph aggregates in the cardiac interstitium, it not only creates a toxic environment for cardiac cells, but also stimulates the interstitium to promote collagen formation and eventual myocardial fibrosis (10). Ishikawa, et al (13) studied different ages and times of onset in patients with myocardial infarction. They showed in early myocardial infarction, the number of lymphatic vessels in the infarcted zone was lower than that in normal myocardium; in the intermediate stage of infarction, the lymphatic vessels could not found in the infarcted zone; in the mid-phase of infarction, few lymphatic vessels emerged, although their density was obviously lower than that of normal myocardium; and in the later period, lymphatic density was significantly increased after myocardial scarring, and this density was greater than normal myocardium. Although there are some differences between their study and ours, the results are similar enough to suggest that reconstitution of lymphatic vessels is of great significance to myocardial functional recovery following myocardial infarction.

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