

Effect of Cardiac Lymph Flow Obstruction on Cardiac Collagen Synthesis and Interstitial Fibrosis

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Summary

The effect of chronic cardiac lymphatic obstruction on the myocardial synthesis of collagen type I and III was investigated in a rabbit model. In the lymphatic obstruction group (n=16), plasma C-terminal propeptide type I procollagen (PICP) and N-terminal propeptide type III procollagen (PIIINP) were elevated at 7, 14 and 30 days after the operation ($p<0.05$). The elevated PICP and PIIINP returned to the pre-operation values 60 days after the operation. The myocardial expression of collagen type I and III mRNA were also enhanced in the lymphatic flow obstruction group. Plasma PICP, PIIINP and myocardial collagen type I and III mRNA remained unchanged in the control group (n=16). We concluded that chronic obstruction of cardiac lymph flow leads to enhanced myocardial collagen synthesis in rabbits. The enhanced collagen synthesis starts within seven days after lymphatic obstruction and subsides after 60 days.

Key words

Cardiac lymph flow • Collagen • mRNA • Rabbits

Introduction

Previous animal studies have shown that acute or chronic obstruction of cardiac lymph flow is associated with significant anatomical and functional changes of the heart. Within several hours of the ligation of cardiac lymphatics, subendocardial edema and hemorrhage, ischemia-type myocardial injuries, myofibrillar degeneration, disruption of Z-band and intercalated disks, and various mitochondrial derangements may occur (Sun and Lie 1977). In animals with chronic cardiac lymphatic

obstruction, endocardial or subendocardial fibrosis (Miller *et al.* 1960, 1963, McKinney 1976) and myxomatous changes in the mitral and tricuspid valves have been demonstrated (Ullal *et al.* 1972).

The mechanism by which lymph flow obstruction is associated with fibrosis within the myocardial interstitial matrix is not entirely clear. Some previous studies suggested that the presence of chronic edema within the interstitium may play a role in myocardial fibrosis (McKinney 1976, Laine and Allen 1991). As the deposition of myocardial interstitial

collagen is a known cause of myocardial fibrosis (Laine and Allen 1991), we hypothesized that the myocardial fibrosis after chronic lymph flow obstruction was due to the enhanced myocardial synthesis and deposition of collagen.

Materials

Surgical preparation of animal model

The study was approved by the Institutional Review Board of Liaocheng City People's Hospital. Thirty-eight New Zealand white rabbits of both sexes (body weight 2.5-3.5 kg) were initially selected for the study. Six animals died during surgical operation. At the end, 32 rabbits with successful surgical preparation were randomly divided into a lymphatic obstruction group (study group, n=16) and sham operated group (control group, n=16).

Animals were anesthetized with intravenous sodium pentobarbitone (30 mg/kg), which was then maintained at 3 mg/kg per hour throughout the surgical procedures. The animals were artificially ventilated with room air at the rate of 60 strokes /min. A left thoractomy was performed in the fourth intercostal space and the heart was suspended in a pericardial cradle.

In the study group, 0.5 ml of 10 % methylene blue was injected into the left and right ventricle near the apex of the heart. Methylene blue stained and clearly marked the lymphatic vessels in the epicardium after the heart surface had been washed with normal saline that was prewarmed to 37 °C. All main lymphatic vessels seen on the epicardium were ligated. The large lymph nodes between the aortic root and pulmonary artery, and between the posterior aorta and the right superior pulmonary artery were also destroyed by electric cautery. The chest was then closed by layers and the animals were returned to the animal house for standard care by the investigators and the animal house curators. Penicillin (average 0.8 million units) was administered intramuscularly daily for a week after surgery to prevent wound infection.

Control group animals underwent the same open-chest surgical procedures as the lymphatic obstruction group, without the ligation of lymphatic vessels and the destruction of lymph nodes.

Hemodynamic monitoring

Body surface ECG and arterial blood pressure were monitored during the experiments. Left ventricular

ejection fraction (EF) was measured by an echocardiograph (HP SONOC-5500, Agilent Technologies, Andover, MA, USA) before and after the operation.

Measurement of collagen type I and type III mRNA in myocardial tissues

Rabbits were euthanized and the hearts were harvested immediately after the euthanasia. Left ventricular tissue (~10 mg) was collected from the regions surrounding the proximal left coronary artery. The collagen type I and III mRNA were measured by an *in situ* hybridization kit (Boster Biotechnology Co., Wuhan, China). Hybridization was performed according to manufacturer's protocols to assess the expression of myocardial collagen type I and type III mRNA. In each group of the animals, *in situ* hybridization was performed in one rabbit after the open-chest surgery but before lymphatic obstruction. It was also performed in a rabbit at 7, 14, 30 and 60 days after the open-chest surgery.

The cardiac tissue was immersed in 4 % formaldehyde polymerisatum (containing 0.1 % diethylpyrocarbonate) for 45 min. Paraffin-embedded serial sections (7 µm thick) were cut and the sections were dried at 80 °C. After rehydration with graded ethanol, the tissue sections were digested in proteinase K (0.5 µg/ml) (concentration 20 mg/ml, specific activity >30 units/mg). in PBS for 10 min at 37 °C. The sections were postfixed with 4 % paraformaldehyde in PBS for 5 min, rinsed twice with 2 mg/ml glycine in PBS for 10 min, and then prehybridized in hybridization buffer at 37 °C for 3 h. Digoxigenin (DIG)-labeled oligonucleotide probes of collagen I and III mRNAs were mixed with hybridization buffer and then applied to each section. The sections were incubated at 37 °C for 24 h.

Tissue sections were washed 4 times with 2× standard saline citrate (SSC) and 50 % formamide at 37 °C for 60 min, 4 times 2×SSC containing 0.075 % Brij-35 at 37 °C for 30 min, 4 times 0.1×SSC containing 0.075 % Brij-35 at 37 °C for 30 min, then 3 times in 0.1 M Tris-HCl pH 7.4, containing 0.15 M NaCl for 5 min at room temperature (22-25 °C). The sections were rinsed with 1 % blocking reagent for 1 h and incubated with alkaline phosphatase-conjugated and DIG antibody for 24 h at 37 °C. The color of the tissue samples was displayed by Nitro Blue Tetrazolium/5-Bromo-4-Chloro-3-Indolyl Phosphate chromogen system (Signet Lab, Dedham, MA, USA) at 37 °C for 3 h. After several times of washing, the sections were counterstained with

hematoxylin, dehydrated with ethanol, rinsed in xylene and mounted with gum for microscopic examination and photography.

Measurement of plasma carboxy terminal propeptide of type I procollagen (PICP) and N-terminal propeptide of type III procollagen (PIIINP)

In each animal, 3 ml of blood was collected from the carotid vein before lymphatic obstruction (the study group) or immediately before the open-chest had been closed (the control group), and 3, 7, 14, 30 and 60 days after the surgery. Serum samples were stored at -80°C . PICP and PIIINP were measured by an enzyme-linked immunosorbent assay (ELISA), using a commercially available test kit (TPI Inc. Washington, USA). Serums (100 μl) and the positive control were added to the precoated wells, then 40 μl anti-rabbit PICP / PIIINP

Biotin and 40 μl anti-rabbit PICP/ PIIINP POD were instilled into the wells which were incubated for 45 min at room temperature. Freshly prepared tetramethyl benzidine solution was added to the wells and incubated for 30 min at room temperature. After blocking the reaction, the absorbance was measured at 450 nm by a microplate reader (Anthos, Salzburg, Austria). CurveExpert1.3 software was used for drawing standard curves and computing the concentrations of PICP, PIIINP.

Statistical analysis

Data were expressed as mean \pm standard deviation. Differences in PICP, PIIINP levels and heart rate, ejection fraction within and between groups were analyzed by Student's t-test. $p < 0.05$ was considered statistically significant.

Table 1. Heart rate changes before and after operation.

	Before	3 d	7 d	14 d	30 d	60 d
Study group <i>n</i> =16	210 \pm 13	304 \pm 45*	315 \pm 46*	302 \pm 40*	217 \pm 21	219 \pm 14
Control group <i>n</i> =16	217 \pm 13	306 \pm 46*	219 \pm 17	224 \pm 18	223 \pm 19	223 \pm 16

* $p < 0.05$

Table 2. Left ventricular ejection fraction (%) before and after operation.

	Before	3 d	7 d	14 d	30 d	60 d
Study group <i>n</i> =16	57 \pm 4	43 \pm 5*	42 \pm 5*	43 \pm 6*	54 \pm 9	57 \pm 4
Control group <i>n</i> =16	54 \pm 6	43 \pm 6*	51 \pm 7	53 \pm 7	54 \pm 5	54 \pm 8

* $p < 0.05$

Table 3. Plasma PICP concentrations ($\mu\text{g/l}$) before and after operation.

	Before	3 d	7 d	14 d	30 d	60 d
Study group <i>n</i> =16	57.1 \pm 6.9	58.4 \pm 6.8	67.0 \pm 6.1*	70.6 \pm 7.6*	74.5 \pm 9.9*	58.2 \pm 6.3
Control group <i>n</i> =16	52.3 \pm 7.2	57.1 \pm 7.1	54.7 \pm 6.0	54.8 \pm 7.7	53.2 \pm 8.1	54.6 \pm 6.8

* $p < 0.05$

Results

General findings

There was an increase in the average heart rate in both groups of animals (Table 1). In the control group, the increased heart rate returned to the pre-operational level seven days after the surgery. In the study group, however, the increased heart rate did not return to the baseline level until two weeks after the operation (Table 2).

In all animals, there was a significant reduction in the left ventricular ejection fraction three days after the surgery (Table 2). The reduced ejection fraction remained low for two weeks in the study group animals but it returned to the baseline level seven days after the operation in the control group.

In the study group, myocardial edema was observed at 3, 7, 14 and 30 days after the operation. Pathological examination with hemotoxylin and eosin stains of the ventricular tissues showed myocardial fibrosis at 14, 30 and 60 days after the surgery. These pathological changes were not demonstrated in the control group animals.

Plasma PICP and PIIINP concentrations

There was no significant difference in the baseline PICP (Table 3) or PIIINP (Table 4) between the study and control groups ($p>0.05$). In the study group, there was a significant increase in plasma PICP at 7, 14 and 30 days after the operation, whereas the plasma PICP remained unchanged in the control group (Table 3).

Plasma PIIINP in the studied group was also elevated 7, 14 and 30 days after surgery. It returned to the baseline level 60 days after the operation. The PIIINP in the control group remained unchanged (Table 4).

Expression of myocardial collagen type I and type III mRNA

In the study group, no evidence of collagen type I mRNA expression was observed in animals without lymphatic obstruction. However, collagen type I mRNA

expression in the ventricular tissues surrounding the left coronary artery was clearly shown at 7, 14 and 30 days after the lymphatic obstruction (Fig. 1). Only small traces of mRNA expression were observed 60 days after the surgery (Fig. 1).

Enhanced collagen type III mRNA expression was also identified at 7, 14 and 30 days after the lymphatic obstruction (Fig. 2). Traces of type III mRNA expression were still evident at 60 days after the surgery (Fig. 2).

There was no evidence of type I or III mRNA expression in the control group of animals.

Discussion

Our study has demonstrated that chronic obstruction of cardiac lymphatic vessels leads to myocardial fibrosis, which is accompanied by a reduction in left ventricular ejection fraction or systolic function. There was an increased expression of collagen type I and III mRNA within 60 days after lymphatic obstruction, and a significant increase in plasma PICP and PIIINP concentrations 30 days after lymphatic obstruction, indicating enhanced collagen synthesis and deposition in interstitial tissues after interference of cardiac lymph flow.

Interstitial fibrosis often occurs in response to the development of interstitial edema (Witte *et al.* 1984). A majority of myocardial tissue fluid leaves the heart through the cardiac lymphatic system, so that a reduction in lymph flow may lead to myocardial edema (Miller 1982). In our rabbit model, ligation of large lymphatic vessels together with the destruction of large cardiac lymph nodes is expected to cause a significant reduction or occlusion of lymph flow (Miller 1985). Although the degree of lymphatic obstruction was not measured in the present study, the surgical procedures resulted in significant myocardial edema within the first four weeks after the operation.

Table 4. Plasma PIIINP concentrations ($\mu\text{g/l}$) before and after operation.

	Before	3 d	7 d	14 d	30 d	60 d
Study group <i>n</i> =16	3.2 \pm 1.0	3.8 \pm 0.9	4.6 \pm 0.9*	5.6 \pm 1.4*	6.3 \pm 0.7*	3.2 \pm 0.7
Control group <i>n</i> =16	3.5 \pm 1.0	3.6 \pm 0.7	3.9 \pm 0.9	4.0 \pm 0.9	3.7 \pm 0.6	3.6 \pm 0.7

* $p<0.05$

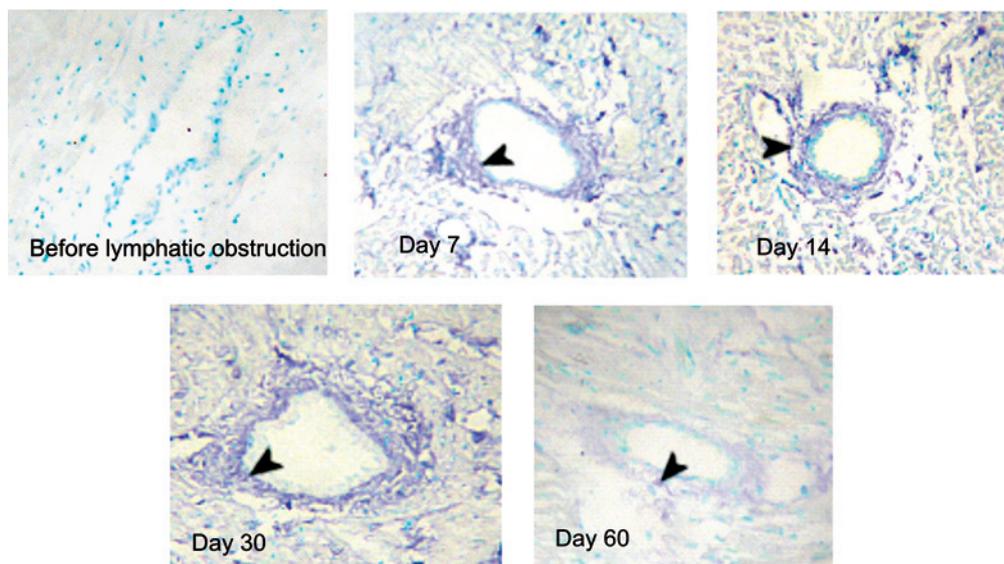


Fig 1. A series of sections of ventricular myocardium harvested from the area of proximal left descending coronary artery shows strong expression of collagen type I mRNA surrounding the artery (arrow) and also in the other areas of the section (stained in purple blue) at 7, 14 and 30 days after lymphatic flow obstruction. There was no type I mRNA expression before the lymphatic obstruction and only limited expression was demonstrated 60 days after the operation.

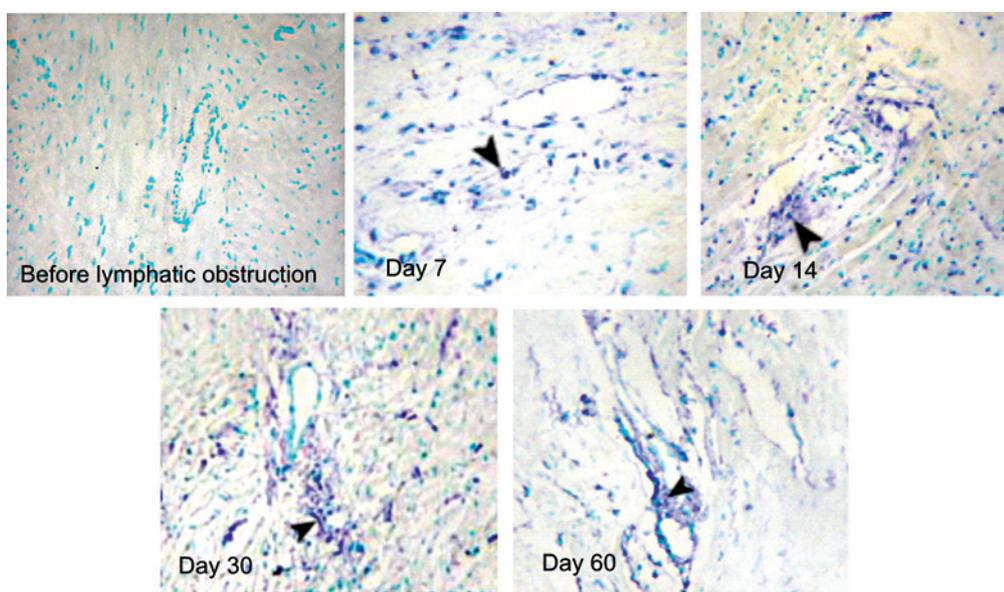


Fig 2. A series of ventricular myocardial tissue sections show the expression of collagen type III mRNA in areas surrounding the artery (arrow) and also in the other parts of the section at 7, 14 and 30 days after lymphatic flow obstruction. A weak collagen type III mRNA expression was also observed 60 days after the operation. There was no type III mRNA expression before the lymphatic obstruction.

Our main interest in the study was whether lymph flow reduction and subsequent myocardial edema would cause myocardial fibrosis *via* collagen synthesis and deposition. To demonstrate the presence of increased level of fibrogenesis in the interstitium, we measured serum PICP and PIIINP, both being indirect indicators of myocardial fibrosis (Diez *et al.* 1995, Querejeta *et al.* 2004). PICP and PIIINP were elevated between day 3 and day 30 of the lymphatic obstruction, suggesting a fibroproliferative reaction or fibrosis.

To confirm the association between fibrosis and collagen synthesis, we also assessed the expression of collagen I and collagen III mRNAs from the ventricular tissues. The expression of both types of mRNA was evident in the myocardium and in the coronary artery

walls between three and 30 days of lymphatic obstruction. A weak expression of both types of genes was also present 60 days after the surgery. The time course of these gene expression coincided with the changes in plasma levels of PICP and PIIINP, suggesting that collagen synthesis and deposition is the main cause of myocardial fibrosis after the lymphatic obstruction.

The signaling pathways between lymphatic obstruction and collagen I and III synthesis are not entirely clear. Proteins in the edematous myocardial tissues may stimulate the deposition of fibrotic materials (Witte *et al.* 1984). However, significant myocardial fibrosis has been identified in both high-protein edema model (e.g. chronic arterial hypertension) and low-protein edema model (e.g. right heart failure) (Laine and Allen

1991). Therefore, proteins in the edematous tissues are not the only triggers for collagen synthesis and deposition. In cultured cells, elevated pressure induces synthesis of collagen by fibroblasts (Riley and Gullo 1988). Myocardial interstitial edema increases interstitial fluid pressure (Laine and Allen 1991), which may in turn stimulate the synthesis and deposition of collagens by fibroblasts.

This study also demonstrated that lymphatic obstruction compromises cardiac function. Although there was also a temporary reduction in left ventricular ejection fraction in the control animals, the ejection fraction recovered seven days after the surgery. In the study group, however, the reduced ejection fraction did not return to baseline values until 14 days after the

surgery. These results suggest that interstitial edema and/or increased collagen synthesis and deposition following lymphatic obstruction can compromise the contractility of the ventricular myocardium.

In conclusion, cardiac lymph flow obstruction in this rabbit model results in myocardial fibrosis by inducing the synthesis and deposition of collagens in the myocardial interstitium. The impairment of ventricular function seen in this animal model is likely due to the myocardial edema and/or fibrosis following lymphatic obstruction.

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