Alteration of extracellular collagen matrix in the myocardium of canines infected with *Dirofilaria immitis*

Jiunn-Shiou Wang a, Kwong-Chung Tung a, Chiu-Chen Huang b, Cheng-Hung Lai c,∗

a Department of Veterinary Medicine, College of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan
b Department of Medical Technology, Yuanpei University of Science and Technology, Hsinchu County, Taiwan
c Department of Biotechnology, Fooyin University, 151 Chiu-Hsueh Road, Ta-Liao Hsien, Kaohsiung Hsien 831, Taiwan

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Abstract

The heart consists of cardiocytes and the interstitial extracellular matrix (ECM), which is made up mainly of collagens. The ECM has been suggested to be important in maintaining the structure and function of the heart. This investigation attempted to elucidate the changes in the ECM collagens in the hearts of canines with dirofilariasis. The ECM collagen fibrils of the heart are grouped into endomysial struts, epimysial weaves, and perimysial coils. In the present study, we used the modified silver impregnation technique to stain paraffin-embedded sections to demonstrate three types of ECM. The results revealed that the ECM content of the heart was significantly reduced in heartworm-infected dogs, and became fragmented and dissociated. In addition, the amounts of collagen in the septum (Sep), RVs and LVs in canines with dirofilariasis (Sep = 11.55 ± 0.65, RV = 12.07 ± 0.59, LV = 11.72 ± 0.62 μg/mg, n = 24) were significantly lower (p < 0.01) than that in the normal canines (Sep = 15.09 ± 0.72, RV = 15.16 ± 0.83, LV = 14.91 ± 0.89 μg/mg, n = 8). These results indicated that heartworm infection induced the remodeling of the extracellular matrix, thus markedly altering the architecture and function of the heart.

Keywords: *Dirofilaria immitis*; Extracellular matrix; Collagen

1. Introduction

The extracellular space of the myocardium contains a collagen network that mainly comprise types I and III fibrillar collagens (Caulfield and Borg, 1979; Eghbali and Weber, 1990; Weber et al., 1994). The quantity and quality of the extracellular collagen is determined by the balance between synthesis and degradation (Tyagi, 1997; Rao and Spinal, 1999). Collagen synthesis is regulated transcriptionally and posttranslationally. Degradation is primarily mediated by matrix metalloproteinases (MMPs), and by endogenous tissue inhibitors (TIMPs) (Cleutjens et al., 1995; Tyagi, 2000; Siwik et al., 2001). The
extracellular matrix (ECM), which is produced primarily by fibroblastic cells and surrounds the cardiac myocytes, preserves the architecture and chamber geometry of the heart (Borg et al., 1996). A disruption or loss of the collagen will result in a reduction in the tensile strength and subsequent muscle bundle slippage. Meanwhile, the three-dimensional network of the ECM comprises three basic levels, including endomysial struts that interconnect adjacent cardiocytes, epimysial weaves that surround cardiocytes, and perimysial coils that course as spring-like structures in the interstitium between cardiocytes (Borg and Caulfield, 1981; Mahbouben and Weber, 1990; Pelouch et al., 1994). Alteration of these structures may lead to the development of heart dysfunction (Weber, 1989; Factor et al., 1991).

*Dirofilaria immitis*, the canine heartworm, resides primarily in the pulmonary arteries and right ventricle. Heartworm infection may cause dilatation of the heart and hypertrophy of the endocardium. The aim of the present study is to investigate the changes of extracellular collagen matrix in the myocardium during the setting of dirofilariasis.

### 2. Materials and methods

#### 2.1. Animals

The study population consisted of 32 mongrel dogs with body weights ranging from 15 to 23 kg. Among them, 24 dogs were diagnosed with moderate to severe dirofilariasis based on the modified Knott’s test (Newton and Wright, 1956), the ELISA kit (Snap™ canine heart PF, IDEXX Laboratory, Westbrook, Maine, USA) and clinical diagnosis (Hoskins, 1996). The dogs were euthanized using intravascular injection of overdose pentobarbital sodium (Nembutal®), Abbott Laboratories, USA), and their hearts were collected. Clinically normal dogs those free of infection with heartworm were used as controls.

#### 2.2. Gross and tissue examination of the heart

The entire heart was removed from the thoracic cavity and washed free of blood. Heart weight, heart weight-to-body weight, and the thickness of left and right ventricles were determined. The specimen was then fixed in 10% phosphate buffered formalin for gross examination. Sections of the left and right ventricles were taken, embedded in paraffin, and stained with hematoxylin and eosin. The stained sections were then examined under a microscope for myocardial abnormalities.

#### 2.3. Modified silver impregnation and histological examination

This study adopted the modified silver impregnation method described by Shyu et al. (1994) and Chiu et al. (1999) to detect myocardial matrix. Briefly, thick sections (25–50 μm) of formalin-fixed and paraffin-embedded cardiac tissue were deparaffinized in xylene and washed in distilled water. The washed sections were immersed in 50% Rio Hortega lithium–silver solution at 60–62 °C for 20 min. The sections were then incubated in an ammonia water-bath (10 drops of 28% ammonia in 50 ml DW) for 2 min, followed by washing with DW for 3 min. After ammonia treatment, the sections were immersed in 0.5% neutral formalin for 1 min and then the excess formalin was washed off with water. The sections were last stained with 0.2% gold chloride for 1 min. After washing and dehydration, the sections were mounted with gelatin. Finally, the collagen matrix of the heart was stained black under light microscopic examination.

#### 2.4. Quantitation of collagen and total protein

A previously described method (López-De León and Rojkind, 1985) was used to quantify the collagen and total protein contents of the paraffin-embedded tissue sections. This method used the selective binding of Sirius red F3BA to collagen protein and Fast green FCF to noncollagen protein when both are dissolved in aqueous saturated picric acid. Briefly, individual slices with a section area of approximately 30–50 mm² were placed in small test tubes and covered with 0.2 ml of saturated picric acid solution that contained 0.1% Sirius red F3BA and 0.1% Fast green FCF. The tubes were incubated at room temperature for 30 min in a rotary shaker, the fluids carefully withdrawn and the sections washed repeatedly with DW until the fluid was colorless. When the sections were treated with sodium hydroxide–methanol, the eluted color was immediately read using a spectrophotometer at 540 and 605 nm.
2.5. Statistics

All data were expressed as mean ± standard error. The Student’s t-test was used to compare the differences between groups. Probability values of below 0.05 were considered significant.

3. Results

The hearts of heartworm-infected dogs became enlarged and rounded in morphology, and the thickness of the ventricle decreased slightly. All three types of collagen matrix including struts, weaves and

Fig. 1. Silver impregnation staining of normal myocardium showing three types of collagen matrix. (A) The struts (arrow) interconnect the cardiocytes. (B) The weaves (arrow) surround the cardiocytes. (C) The coils (arrow) in the interstitium between cardiocytes. In heartworm-infected dogs, the number of struts was markedly reduced (D). The number of weaves and coils were similarly significant decreased and became fragmented and dissociated (E and F).
coils were found in the ECM of normal canine heart tissues by using the modified silver impregnation. Endomysial struts interconnected the cardiocytes, epimysial weaves encircled the sarcolemma of cardiocytes, and perimysial coils formed helix structure in the interstitium (Fig. 1A–C). In heartworm-infected dogs, the number of endomysial struts was significantly decreased in number (Fig. 1D). The weaves and coils displayed a similar significant decrease in number, and became fragmented and dissociated (Fig. 1E and F). These phenomenon were most prevalent in the right ventricle.

In the present study, we determined the quantity of collagen and total protein content in paraffin-embedded tissue sections from the hearts of clinically normal dogs (n = 8) and heartworm-infected dogs (n = 24). The collagen-to-total protein (µg/mg) content in heartworm-infected dogs was significantly lower than that in clinically normal dogs (p < 0.01); septum, 11.55 ± 0.65 versus 15.09 ± 0.72; RV, 12.07 ± 0.59 versus 15.16 ± 0.83; and LV, 11.72 ± 0.62 versus 14.91 ± 0.89 (Table 1).

4. Discussion

The interstitium of the myocardium includes fibrillar connective tissue, various cells such as fibroblasts and plasma cells, and a gel-like ground substance comprising glucosaminoglycans and glycoproteins (Borg and Caulfield, 1981). Myocytes represent only one-third of the number of cells, but their volume accounts for over two-thirds of the myocardium. Meanwhile, fibroblasts may represent as many as two-thirds of the cells (Zak, 1973). The fibroblasts are the principal type of cell responsible for the production and deposition of the ECM in the interstitium, especially fibrillar collagen types I and III, which interconnect the cellular components of the heart (Kanekar et al., 1998). The functions of cardiac interstitium include providing a structure that supports and tethers cardiac myocytes and vessels; maintaining a defense mechanism against invasion by foreign proteins, bacteria or viruses; aiding in the nutrition of myocytes; and providing a lubricant for contracting myocytes (Weber, 1989).

Normal cardiocytes have three main types of the ECM, including struts, weaves, and coils, which are important to maintaining the physiological function of the heart (Robinson et al., 1986; Factor and Robinson, 1988). Chronic alterations in blood volume/pressure load induce myocardial and vascular remodeling and develop hypertrophy to accumulate the workload. Chronic increase in load for an extended period of time leads to stiffness in the muscle, hypertrophic cardiomyopathy, ventricular dysfunction and heart failure (Conrad et al., 1991). In left ventricle pressure overload hypertrophy, an accumulation and enhanced dimension of collagen fibers, together with their realignment relative to muscle cells have been observed (Weber et al., 1988). In the pigs with hypertrophic cardiomyopathy, the amounts of collagen in the ventricle were significant increased than that in normal pigs (Chiu et al., 1999). In the dilated failing ventricle secondary to rapid pacing, there is a structural remodeling of the cardiac interstitium and intramyocardial coronary arteries with interstitial edema and collagen fibers degradation (Weber et al., 1990).

*Dirofilaria immitis* is one of the most important parasites of canine, which resides primarily in the pulmonary arteries and right ventricle and can severely affect canine health. Dogs with heartworm disease gradually develop chronic pulmonary hypertension and right ventricle failure. Rawlings and Lewis (1977) demonstrated that the right ventricle of dogs appears to dilate in the response to *D. immitis* infestation. The present study revealed that the ECM

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<th>Collagen (µm/mg of tissue protein)</th>
<th>Heartworm-infected dogs (n = 24)</th>
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<tr>
<td><strong>Septum</strong></td>
<td>15.09 ± 0.72</td>
<td>11.55 ± 0.65</td>
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<tr>
<td><strong>Right ventricle</strong></td>
<td>15.16 ± 0.83</td>
<td>12.07 ± 0.59</td>
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<tr>
<td><strong>Left ventricle</strong></td>
<td>14.91 ± 0.89</td>
<td>11.72 ± 0.62</td>
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*p < 0.01, value are mean ± S.E., differences between groups were evaluated by Student’s t-test.*
content of the heart was significantly reduced in heartworm-infected dogs and became fragmented and dissociated when the silver impregnation stain was employed. The decreased collagen matrix may be an important factor contributes to the dilatation of the ventricle, thereby markedly affecting the systolic and diastolic functions of the heart.

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**References**


