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Original article

Early dystrophin disruption in the pathogenesis of experimental chronic Chagas cardiomyopathy

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Abstract

Chronic Chagas cardiomyopathy evolves over a long period of time after initial infection by *Trypanosoma cruzi*. Similarly, a cardiomyopathy appears later in life in muscular dystrophies. This study tested the hypothesis that dystrophin levels are decreased in the early stage of *T. cruzi*-infected mice that precedes the later development of a cardiomyopathy. CD1 mice were infected with *T. cruzi* (Brazil strain), killed at 30 and 100 days post infection (dpi), and the intensity of inflammation, percentage of interstitial fibrosis, and dystrophin levels evaluated. Echocardiography and magnetic resonance imaging data were evaluated from 15 to 100 dpi. At 30 dpi an intense acute myocarditis with ruptured or intact intracellular parasite nests was observed. At 100 dpi a mild chronic fibrosing myocarditis was detected without parasites in the myocardium. Dystrophin was focally reduced or completely lost in cardiomyocytes at 30 dpi, with the reduction maintained up to 100 dpi. Concurrently, ejection fraction was reduced and the right ventricle was dilated. These findings support the hypothesis that the initial parasitic infection-induced myocardial dystrophin reduction/loss, maintained over time, might be essential to the late development of a cardiomyopathy in mice.

Keywords: Chronic Chagas cardiomyopathy; Dystrophin; Chagas disease; Dystrophinopathy; *Trypanosoma cruzi*

1. Introduction

One of the most intriguing aspects of chronic Chagas cardiomyopathy (CCC) is that it evolves over a long period of time after initial infection by the protozoan *Trypanosoma cruzi* (*T. cruzi*) [1–4]. Chagas disease is characterized by three phases: acute, indeterminate, and chronic. The heart is the most severely and frequently involved organ. A mild to severe acute myocarditis characterizes the cardiac involvement during the acute phase, with predominant mononuclear infiltrate around ruptured pseudocysts of parasites, and intense parasitism of myofibers that spontaneously subsides after 2–3 months in most cases. The indeterminate phase is a prolonged, clinically silent period – 10–30 years – that follows the acute phase. The patients present serological and/or parasitological evidence of infection with no symptoms or only minor disturbances of cardiac rhythm. The chronic phase evolves from the indeterminate phase in 10–30% of the cases in humans. Grossly, the heart is usually enlarged due to dilatation and hypertrophy, with 60–70% of patients presenting the characteristic apical aneurysm. Microscopically, a progressive, destructive and reparative chronic fibrosing myocarditis characterizes the chronic phase. Parasite antigens or, rarely, nests of *T. cruzi* are observed within cardiomyocytes and remain there as a result of host specific defense [1,3,5–7]. However, the mechanisms associated with the establishment/

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maintenance of distinct clinical outcomes of Chagas disease appear to be complex and the transition to the chronic form remains to be elucidated.

Similar to CCC in humans, cardiac complications due to cardiomyopathy appear later in life in Duchenne muscular dystrophy and Becker muscular dystrophy, the most common X-linked recessive disorders resulting from mutations in the dystrophin gene that lead to an absence of or defect in the protein dystrophin in striated muscles. Dystrophin and associated glycoproteins form the so-called dystrophin glycoprotein complex, which contributes to cell shape, mechanical resistance, and contraction and force generation in cardiomyocytes [8]. It is known that mutations in the dystrophin gene result in membrane damage and necrosis that is associated with severe muscle degeneration and massive chronic inflammatory infiltrate [9]. Dystrophin reduction has been linked to end-stage cardiomyopathies and has been proposed as a common route to the induction of cardiomyopathy and heart failure [10,11]. Furthermore, dystrophin loss has been observed in different forms of acquired cardiomyopathies, such as post-viral myocarditis caused by Coxsackie virus B [12] and septic cardiomyopathy [13].

The development of cardiomyopathy in both Chagas disease and congenital dystrophinopathies occurs decades after infection or birth in humans, respectively. The present study tested the hypothesis that cardiac dystrophin levels are decreased during the early phase of the experimental infection by *T. cruzi* in mice and are maintained at low levels up to 100 days post infection (dpi); thus, explaining, in part, the late development of cardiomyopathy.

2. Materials and methods

2.1. Animals and experimental infection

The Brazil strain of *T. cruzi* was maintained in C3H mice (Jackson Laboratories, Bar Harbor, Maine, USA). Male CD1 mice (Charles River, Wilmington, MA, USA) were infected intraperitoneally at 10 weeks of age with 5×10^4 trypomastigotes. All mice were housed in the Institute for Animal Studies of the Albert Einstein College of Medicine and all protocols were approved by the Institutional Animal Care and Use Committee (Albert Einstein College of Medicine, New York) and by the Committee on Animal Research of the Faculty of Medicine of Ribeirão Preto (University of São Paulo, Brazil). The levels of parasitemia were evaluated using 5 μl of blood obtained from the tail vein of infected mice at days 15, 20, 30, 40, 60, 80 and 100 dpi. The mortality was evaluated throughout the experimental period.

2.2. Histopathological and morphometric analysis

Control and infected mice were sacrificed 30 and 100 dpi. These time points were chosen because 30 dpi is the peak of mortality associated with intense acute myocarditis with an inflammatory infiltrate mainly composed of lymphomononuclear cells and striking tissue parasitism and 100 dpi with cardiomegaly and mild to moderate chronic myocarditis characterized by infiltration of the interstitial space by mononuclear and spindle cells, interstitial fibrosis and the absence of tissue parasitism [14–16]. The hearts were rapidly removed, rinsed in ice-cold 0.9% NaCl solution and fixed in neutral 10% formalin for histological study or immediately frozen in liquid nitrogen-cooled isopentane for immunofluorescence study. Both ventricles from each heart were isolated and cut into two fragments by a mid-ventricular coronal section.

For histopathological study ($n = 6$/day/group), the samples were dehydrated, clarified, embedded in paraffin, stained with hematoxylin and eosin and picrosirius red and examined by light microscopy. The tissue sections stained with hematoxylin and eosin were used to evaluate the intensity of inflammation, the presence of amastigote nests and tissue damage. The slides stained with picrosirius red ($n = 6$/day/group) were used to evaluate fibrosis through collagen quantification.

For morphometric analysis ($n = 6$/day/group), the Leica QWin software (Leica Imaging Systems Ltd., Cambridge, England) in conjunction with a Leica microscope, videocamera, and an online computer was used. The number of inflammatory cells was determined by counting the number of mononuclear rounded interstitial cells (to exclude the spindle shaped fibroblastic cells) in the myocardium of the right and left ventricles in 10 microscope fields (40×1.6 magnification) per ventricle of each animal. To estimate the volume fraction (%) of fibrosis in picrosirius red-stained sections of the right and left ventricles, 10 microscope fields (400× magnifications) per ventricle of each animal were measured under polarized light with the QWin software. Measurements were made by a skilled observed blinded to the groups.

2.3. Immunofluorescence

For immunofluorescence microscopy, 5 μm frozen sections ($n = 6$/day/group) were transferred to silane-coated slides and fixed in cold acetone for 10 min. Immunolabeling was performed using primary antibody to dystrophin (rabbit polyclonal antibody anti-dystrophin, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, dilution 1:200), diluted in 1% BSA and incubated overnight at 4 °C. Immunolabeling was performed using goat anti-rabbit fluorochrome-conjugated secondary antibody (FITC) (Vector Laboratories Inc., Burlingame, CA, USA) diluted 1:200 in HEPES 0.01 M and incubated for 1 h at room temperature. Omission of the primary antibodies served as negative control. Some sections were also labeled with phalloidin complexed to rhodamine (Alexa Fluor 594 phalloidin, Molecular Probes, Eugene, OR, USA) for visualization of actin. The nuclei were labeled with DAPI (Molecular Probes).

2.4. Western blotting

To determine the amount of dystrophin in control and chagasic hearts ($n = 5$/day/group), homogenates of the left and right ventricles were analyzed by immunoblotting at 30 and