INSTITUT PASTEUR

Microbes and Infection 10 (2008) 21–28

www.elsevier.com/locate/micinf

Original article

Trypanosoma cruzi induces changes in cardiac connexin43 expression☆

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Received 15 August 2007; accepted 25 September 2007
Available online 2 October 2007

Abstract

Gap junction proteins (connexins) are required for myocardial function, since they allow intercellular transmission of current carrying ions and signaling molecules. Previous studies demonstrated that rat cardiac myocytes infected with *Trypanosoma cruzi* lost gap junctional communication and decreased automaticity. We infected mouse cardiac myocytes with trypomastigotes of the Y strain of *T. cruzi* and observed alterations in connexin43 (Cx43) distribution. One hour post infection Cx43 levels were significantly increased. However, at longer time points post infection there was a significant loss of Cx43 staining in membranes of infected cardiac myocytes. Interestingly, there was also a significant reduction in myocardial Cx43 protein levels during acute infection. These data indicate that *T. cruzi* infection alters Cx43 expression both *in vitro* and *in vivo*. Disruptions in Cx43 may contribute to the pathogenesis of cardiac electrical alterations observed in *T. cruzi* infection.

Keywords: *Trypanosoma cruzi*; Chagas' disease; Cardiac myocytes; Connexin43; Gap junctions

1. Introduction

Trypanosoma cruzi is the causative agent of Chagas' disease (American Trypanosomiasis). It is the major cause of heart disease in endemic areas of Mexico, Central and South America. Ten to 30% of infected individuals eventually develop clinically apparent heart disease including dilated cardiomyopathy, congestive heart failure, dysrhythmias and thrombo-embolic events.

This parasite has a complicated life cycle involving a mammalian host and an insect vector [1]. During *in vitro* infection of mammalian cells (host cells), *T. cruzi* alters intracellular calcium levels both in non-excitable cells [2] and in cardiac myocytes [3,4]. The parasites enter cells through a mechanism involving the formation of a parasitophorous vacuole, which arises from the fusion of lysosomes and endocytic vesicles [5]. After invasion, trypomastigotes escape from the vacuole and remain free within the cytoplasm where they differentiate to the multiplicative amastigote form [6]. Amastigotes multiply by binary fission and eventually escape the host cell as trypomastigotes capable of infecting adjacent uninfected cells and spreading by a hematogenous route to distant tissues resulting in the infection of new cells.

Studies of alterations in cardiac myocytes during *in vitro* infection with *T. cruzi* indicate that the parasite is capable of impairing host cell functioning through alterations in cell-cell

☆ All authors have no commercial or other association that might pose a conflict of interest. This work was supported by FIOCRUZ/FAPERJ, PAPES IV–FIOCRUZ, CNPq and NIH AI-052739 (HBT), HD32573 (DCS), Daniel Adesse is a PhD student and was supported by IOC–FIOCRUZ and a Fogarty International Training Grant (D43TW007129).
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doi:10.1016/j.micinf.2007.09.017

communication [7]. Such an effect is of particular importance in the heart where maintenance of synchronous contractions requires functional gap junctions (see Refs. [8,9], for reviews).

Gap junction channels are composed of the connexin family of transmembrane proteins that assemble as end to end alignments of hexameric connexin subunits, thereby forming intercellular conduits for current-carrying ions and molecules $M_r < 1000$ Da such as Ca^{2+}, IP_3 and cyclic AMP. The connexin gene family in mammals includes more than twenty isoforms encoded by separate genes [10] and such isoforms are named according to the molecular weight (in kDa) of the protein predicted from its cDNA [11]. Gap junction channels are critical in maintaining cardiac homeostasis by allowing the free flow of ions and metabolites between cardiac myocytes. This contributes to the synchronized contraction of and signal exchange throughout the tissue. Connexin43 (Cx43) is the most abundant gap junction protein in ventricular myocytes, being localized at intercalated disks in normal myocardium (see Ref. [8]). Cx43 function and intercellular distribution is affected by its phosphorylation and other factors including intracellular pH and calcium concentration and protein-protein interactions [12]. In diverse cardiac disease states, such as infarct and ischemia, significant remodeling of the distribution of Cx43 occurs, resulting in disorganization of normal microconduction pathways and arrhythmias [13].

Since gap junctional communication is important in normal cardiac conduction and the report that intercellular communication and Cx43 distribution are altered in *T. cruzi*-infected rat cardiac myocytes [7], we examined the expression and distribution of Cx43 in widely used *in vivo* and *in vitro* mouse models of infection. The present study characterizes such changes in cultured cardiac myocytes during initial infection and during the intracellular cycle of this parasite and demonstrates that Cx43 expression is down-regulated in the hearts of infected mice. These alterations in Cx43 may contribute to the cardiac dysrhythmias observed in the acute phase of *T. cruzi* infection.

2. Materials and methods

2.1. Antibodies and reagents

Rabbit polyclonal antibody against Cx43 (C6219, amino acids 363–382), 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI); Phenylmethanesulfonyl fluoride (PMSF); 1,4-Diazabicyclo[2.2.2]octane (DABCO); Ethylene Diamine Tetraacetic Acid (EDTA); Sodium Bicarbonate ($NaHCO_3$); Sodium Orthovanadate (Na_3VO_4); Ponceau Solution; Polyethylene glycol sorbitan monolaurate (Tween 20), Triton X-100 and Bovine Serum Albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, IL, USA). Rabbit polyclonal antibody against Cx43 181A (amino acids 346–360) was kindly provided by Professor Elliot Hertzberg, Department of Neuroscience, Albert Einstein College of Medicine. Secondary goat anti-rabbit IgG AlexaFluor 594 and Phalloidin AlexaFluor 488 were purchased from Molecular Probes (Invitrogen, Carlsbad, CA). Monoclonal anti-rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was obtained from RDI Division of Fitzgerald Industries Intl.

For cardiac myocyte isolation and cultivation, trypsin was obtained from Difco Laboratories, type II collagenase from Worthington Biochemical Corporation (Lakewood, NJ), and Fetal Bovine Serum (FBS), L-glutamine, penicillin, streptomycin and Dulbecco's Modified Eagle's Medium (DMEM) from Sigma-Aldrich.

2.2. Primary cultures of mouse cardiac myocytes

Hearts from Swiss Webster mouse embryos (18–21 days of gestation) were harvested and digested with collagenase and trypsin using the method described by Meirelles [14]. Isolated cells were plated in gelatin coated dishes and maintained in DMEM medium supplemented (DMEMs) with 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin, 2% L-Glutamine, 2% Chick Embryo Extract, 10% $CaCl_2$ 25 mM [4,14]. For immunoblotting, 2×10^6 cells were plated on 60 mm cell culture plastic dishes (Corning Inc., Corning, NY) and for Giemsa microscopy experiments, 5×10^5 cells were plated on glass coverslips in 24-well plates.

2.3. Infection with Trypanosoma cruzi (Y strain)

Trypomastigotes of the Y strain (MHOM/BR/1950/Y) were obtained from infected Vero cell cultures. The parasites in the supernatant were collected and centrifuged at 3000 rpm for 30 min at 4 °C. Cardiac myocytes were infected after 72 h of plating. The multiplicity of infection was 20:1 (parasites: host cell) and parasites were diluted in DMEM without FBS in a final volume of 300 µl for 24-well plates and 1 ml for 60 mm dishes. After two hours of interaction, extracellular parasites were washed out and fresh supplemented DMEM was added. For subsequent days, medium was replaced every 24 h. Cardiac myocytes were fixed and stained with Giemsa stain for assessment of intracellular parasitism as previous described [15].

2.4. Immunofluorescence

Cells were washed with PBS and fixed with 4% Paraformaldehyde for 5 min at 20 °C at desired time points (1 and 72 hpi). After washes in PBS, cells were permeabilized with 0.5% Triton and non-specific staining was blocked with 4% BSA. Primary anti-Cx43 antibody (Sigma) was incubated overnight 4 °C, after which cells were washed and incubated with secondary polyclonal goat anti-rabbit Alexa Fluor 594 antibody (Invitrogen) for 1 h at 37 °C. F-actin filaments were stained with Alexa Fluor 488 Phalloidin (Invitrogen) for 30 min at 37 °C and DNA was stained with DAPI. Images were acquired with a Olympus Laser Scanning Confocal Microscope.

2.5. Immunoblotting

At desired intervals after infection (1, 2, 6, 24, 48 and 72 h post infection), cells were washed 3 times with PBS and