Microbial Pathogenesis 73 (2014) 47–52

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Cardiopathogenic mediators generated by GATA4 signaling upon co-activation with endothelin-1 and *Trypanosoma cruzi* infection

Cristina S. Rigazio, Matías Hernández, Ricardo S. Corral*

Servicio de Parasitología-Chagas, Hospital de Niños "Dr. Ricardo Gutiérrez", Gallo 1330, 1425EFD Ciudad de Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 7 March 2014
Received in revised form
26 May 2014
Accepted 3 June 2014
Available online 11 June 2014

Keywords:
Chagas cardiomyopathy
Trypanosoma cruzi
Endothelin-1
GATA4

ABSTRACT

Trypanosoma cruzi (Tc), the etiological agent of Chagas disease, triggers multiple responses in the myocardium, a central organ of infection and pathology in the host. Parasite-driven induction of diverse regulators of cardiovascular function, including the vasoconstrictor endothelin-1 (ET-1), the inducible form of nitric oxide synthase (iNOS) and the B-type natriuretic peptide (BNP), has been linked to the development of severe chagasic cardiomyopathy. Our current goal was to analyze the participation of the zinc finger transcription factor GATA4, critically implicated in pathological cardiac hypertrophic response, in the generation of key mediators involved in the pathogenesis of Tc-elicited heart dysfunction. In this study, we found that the combined effects of Tc and ET-1 on atrial myocytes promoted the protein expression, phosphorylation and DNA-binding activity of GATA4, leading to augmented protein levels of iNOS and increased nitric oxide release. Moreover, Tc- and ET-1-co-activation of cardiomyocytes resulted in enhanced GATA4-dependent secretion of BNP. Accordingly, mice with chronic chagasic cardiomyopathy showed increased expression of GATA4, iNOS and BNP at inflammatory lesions in cardiac muscle. Our findings support a role for the GATA4 signaling pathway in the myocardial production of pathogenic mediators associated with Chagas heart disease, and may help define novel therapeutic targets.

1. Introduction

Chagas disease, caused by the hemoflagellate protozoan *Trypanosoma cruzi*, is one of the most serious public health and socio-economic problems in Latin America. The overall prevalence of chronic infection is about 8–10 million cases, with 50,000 people estimated to die from the disease each year [1]. In recent decades, this parasitosis has spread around the globe through human migration to non-endemic areas [2]. Upon *T. cruzi* infection, individuals undergo an acute phase which displays frequently as a non-apparent form with a few or no symptoms. Thereafter, the majority of chagasic subjects enter into an asymptomatic, indeterminate stage, which lasts throughout life. The remaining 20–30% of chronically infected patients develop clinical complications, typically years or decades after infection. Chronic cardiomyopathy is the most common and severe manifestation of Chagas disease, causing congestive heart failure, arrhythmias and conduction abnormalities, which often result in stroke and sudden death. This type of dilated cardiomyopathy is associated with chronic inflammation and fibrosis, cardiac hypertrophy and thrombo-embolic events [3].

The pathogenesis of *T. cruzi*-driven cardiomyopathy is still matter of intense debate. However, three main pathogenic mechanisms have been identified: cardiac dysautonomy, inflammatory/immunological tissue damage and disorders of the microvascular circulation [4]. Among other factors, elevated levels of the vasoactive peptide endothelin-1 (ET-1) play a pivotal role in the development of Chagas heart disease contributing to vascular injury, cardiac remodeling and enhanced liberation of inflammatory agents [5]. Recently, we found that the combined effect of *T. cruzi* infection and ET-1 activates the Ca^{2+}/calcineurin (Cn)/nuclear factor of activated T cells-c4 (NFATc4) signaling pathway in atrial myocytes, leading to cyclooxygenase-2 (Cox-2) overexpression and increased eicosanoid release [6].

The discovery of cooperation between the NFATc4 protein and the zinc finger transcription factor GATA binding protein 4 (GATA4), critically involved in pathological cardiac hypertrophic response, has revealed the importance of Ca^{2+} signaling in the activation of this GATA family member [7]. We therefore hypothesized that, upon ET-1 treatment and parasite infection, stimulation of the intracellular Ca^{2+}-dependent cascade may trigger GATA4

* Corresponding author. Tel./fax: +54 11 4963 4122.
E-mail address: ricardocorral56@hotmail.com (R.S. Corral).

http://dx.doi.org/10.1016/j.micpath.2014.06.001
0882-4010/

Glossary

Tc	Trypanosoma cruzi
ET-1	endothelin-1
Cn	calcineurin
NFAT	nuclear factor of activated T cells
Cox-2	cyclooxygenase-2
GATA4	GATA binding protein 4
phospho-GATA4	phosphorylated form of GATA4
Ser-105	amino acid serine at position 105 in the GATA4 protein
BNP	B-type natriuretic peptide
iNOS	inducible form of nitric oxide synthase
PMA	phorbol 12-myristate 13-acetate
siRNA	small interfering RNA
Wt	wild-type
Mut	mutant

phosphorylation in cardiac muscle cells. GATA4 downstream target genes include B-type natriuretic peptide (BNP) and inducible nitric oxide synthase (iNOS), both implicated in Chagas pathogenesis [8,9]. Nevertheless, contribution of GATA4 activity to *T. cruzi*-elicited myocardial dysfunction remains unexplored so far. Our current goal was to examine whether the cooperative action of the pathogen and ET-1 on atrial myocytes leads to GATA4-regulated induction of critical mediators of cardiovascular pathology in Chagas disease.

2. Materials and methods

2.1. Ethics statement

The protocol of this study was reviewed and approved by the Research & Teaching Committee from Hospital de Niños "Dr. Ricardo Gutiérrez" (Buenos Aires City Government, Argentina).

2.2. Trypanosoma cruzi infection of cardiac myocytes

Mouse HL-1 cardiomyocytes were cultured as described previously [10]. Cells (5×10^5) were infected with *T. cruzi* trypomastigotes (cell:parasite ratio 1:5), Tulahuén strain, routinely propagated in Vero cells. In some experiments, cell cultures were starved for 18 h and then treated with endotoxin-free 1 nM ET-1 (Sigma–Aldrich) for 2 h before infection. Cultures were rinsed to remove free parasites and further incubated at 37 °C under 5% CO_2 for the indicated times.

2.3. Immunoblot analysis

Immunoblotting was carried out as described elsewhere [11]. HL-1 cells were stimulated for 2 h with ET-1 and/or infected with *T. cruzi* for 15 min (GATA4 analysis) or 3 h (iNOS expression). Phorbol 12-myristate 13-acetate (PMA, Sigma–Aldrich, 0.5 nM) was used as standard stimulus for GATA4 induction. Untreated samples were included as controls. Myocytes were disrupted and solubilized whole cell extracts (50 μg) were electrophoretically separated in 10% sodium dodecyl sulfate-polyacrylamide gels, and transferred to nitrocellulose filters. The membranes were probed 2 h at 37 °C with rabbit polyclonal antibodies (1 μg/ml) against dephosphorylated murine GATA4 (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated (Ser-105)-GATA4 (phospho-GATA4, Thermo Scientific Pierce, Waltham, MA), or iNOS (Santa Cruz Biotechnology), and mouse monoclonal antibody against α-tubulin (0.5 μg/ml, Sigma–Aldrich). The filters were washed and incubated with the corresponding secondary antibody linked to horseradish peroxidase (Thermo Scientific Pierce) and the stained bands were visualized by a chemiluminescent peroxide substrate (GE Healthcare, Pittsburgh, PA). Band intensity was analyzed using NIH Image J software [12].

2.4. Preparation of nuclear extracts

Nuclear extracts from stimulated (2 h)/infected (3 h) HL-1 cardiomyocytes were prepared as described previously [13]. Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA).

2.5. GATA4 DNA-binding activity

Nuclear extracts (15 μg) were applied to a GATA4 transcription factor assay kit (TransAM, Active Motif, Carlsbad, CA) according to the manufacturer's instructions [14]. Activated GATA4 present in nuclear extract binds specifically to an oligonucleotide containing the consensus binding site 5′-AGATAA-3′ immobilized on the microplate. In some experiments, a competitor oligonucleotide (wild-type or mutant) was added to confirm specific reactivity. The GATA4 antibody recognizes an epitope on the protein that is accessible upon DNA binding. Addition of a secondary peroxidase-conjugated antibody and the corresponding substrate provided a sensitive colorimetric readout quantified by spectrophotometry.

2.6. Small interfering RNA (siRNA)-mediated knockdown

HL-1 cardiomyocytes were transfected with 40 pmol of GATA4-specific Stealth siRNA (Invitrogen™, Life Technologies, Carlsbad, CA) or scramble siRNA (control) for 48 h using Lipofectamine 2000 (Invitrogen™) following the instructions of the supplier. This protocol has been proved to efficiently interfere with GATA4 gene expression [15].

2.7. Quantification of cardiomyocyte-derived soluble mediators

For BNP measurements, 24-h supernatants from ET-1-stimulated and/or *T. cruzi*-infected HL-1 cells were analyzed by ELISA (Kamiya Biomedical, Seattle, WA) according to the manufacturer's instructions. The assay sensitivity was 5.3 pg/ml. Further, nitrite accumulation in the culture supernatants was used as an indicator of NO production and was determined by the Griess reaction with sodium nitrite as a standard, as reported previously [16]. Supernatants (50 μl) were incubated, in the dark and at room temperature, with an equal volume of Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthylethylenediamine dihydrochloride, 2.5% H_3PO_4). The absorbance at 540 nm was read 10 min later.

2.8. Chronic Trypanosoma cruzi infection of mice

Eight-week-old female BALB/c mice were infected intraperitoneally with 25 Tulahuén strain trypomastigotes. At 100 days post-infection, hearts were removed and sectioned. Immunohistochemical analysis of formalin-fixed, paraffin-embedded cardiac muscle specimens from infected and uninfected mice was performed as described previously [17]. BNP immunostaining was accomplished using a rabbit polyclonal antibody specific to the mouse peptide (Bioss, Woburn, MA).