Fibrosis and Hypertrophy Induced by Trypanosoma cruzi in a Three-Dimensional Cardiomyocyte-Culture System

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Cardiac damages caused by in vivo infection with Trypanosoma cruzi are still not fully clarified. Here we describe for the first time an in vitro model of fibrosis, hypertrophy, and remodeling induced by T. cruzi in cardiomyocyte spheroids (cardiac microtissues). In this new 3-dimensional system, cardiac spheroids showed spontaneous contractility, with typical cardiac morphology and production of extracellular matrix components. There were 4- and 6-fold increases, respectively, in the area and the volume of T. cruzi–infected cardiomyocytes and whole microtissues, together with a 50% reduction of the cell population. Immunofluorescence showed increased expression of fibronectin, collagen IV, and laminin in the microtissues 144 h after infection. T. cruzi infection induced an increase in both the cellular area and the extracellular matrix components in cardiac spheroids, which contributed to an increase in total microtissue volume, making this a powerful 3-dimensional in vitro model for the study of cardiac-tissue hypertrophy, fibrosis, and remodeling.

Cardiac remodeling (CR) is characterized by changes in myocardial structures, in response to mechanical overload or cardiac injury. CR can be adaptive or can result from phenotypic modifications, such as fibrosis, during clinical conditions that can alter myocardial gene expression [1].

Chagas disease, which is caused by the protozoan Trypanosoma cruzi, is endemic in Latin America, affecting 16–18 million people, and 90 million are at risk of infection [2]. This disease causes chagasic cardiomyopathy, a progressive disease in which patients present with myocarditis, fibrosis, and myocardial hypertrophy [3].

Recently, it has been shown that cardiomyocytes play an active role in inflammation in response to T. cruzi, because, during infection by this organism, cardiac cells express tissue-necrosis factor α, interleukin 1β [4, 5] and tumor-growth factor β (TGFβ) [6], possibly contributing to the genesis of hypertrophy and fibrosis in vivo. Persistence of infection, presence of parasite antigens at the inflammatory site, and/or autoimmune process are possible causes of the pathological lesions in the heart tissue of infected patients [7–9].

2-Dimensional (2D) monolayer culture systems have been the method of choice in studies of the interaction between T. cruzi and cardiomyocytes and, by providing details on this relationship, have answered many questions concerning alterations in host-cell and parasite physiology [5, 6, 10–20]. However, 2D cultures fail to mimic the CR caused by fibrosis and hypertrophy observed in vivo during chagasic cardiomyopathy.

Bioengineering, which has emerged during the past decade, has focused on cell cultivation by 3D systems, because of the similarity of their results to those of tissue
morphology and because they provide a suitable environment for cellular interactions. Cells in 3D cultivation have greater similarity to the in vivo situation, in terms of gene expression, proliferation, differentiation, and apoptosis. Although monolayer culture systems have contributed to important advances, they are limited in providing clinically relevant information [21, 22].

The aim of the present study was to reproduce, in vitro, conditions related to chagasic cardiomyopathy, by using a 3D cultivation system to infect cardiomyocytes (cardiac spheroids) with *T. cruzi*. We demonstrate that this parasite successfully invades the cardiac spheroids and completes its intracellular cycle. Furthermore, we found that infection induced an increase and redistribution of extracellular matrix (ECM) components such as fibronectin, collagen, and laminin, which was followed by an increase in both the cellular area and the total volume of the cardiac spheroids.

**MATERIALS AND METHODS**

**Antibodies and reagents.** Rabbit polyclonal antibodies against fibronectin and laminin, 4',6-diamidino-2-phenylindole

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**Figure 1.** Ultrastructural aspects of the intracellular cycle of *Trypanosoma cruzi*, as shown by scanning (A–C) and transmission (D–F) electron microscopy. After seven days of culture, cardiac spheroids were infected with *T. cruzi* trypomastigotes. A, Surface of noninfected microtissue, with round cells on a flat surface. B, High magnification of noninfected microtissue, showing more detail of the surface of cardiomyocytes with membrane projections. C, Release of a trypomastigote (arrow). Note the damaged host cell exposing intracellular structures (*) and a preserved area (+). Proliferative amastigote forms (*) observed 48–72 h postinfection display bar-shaped kinetoplasts. D, Differentiated cardiomyocytes, showing intercellular junctions (arrowhead), myofibrils (MF) close to mitochondria (Mt), and sarcoplasmic reticulum (SR). E, Beginning of the infection process, showing a trypomastigote (P) with the typical basket-shaped kinetoplast inside a parasitophorous vacuole (PV). F, Proliferative amastigote forms (*) observed 48–72 h postinfection, displaying bar-shaped kinetoplasts.
dihydrochloride (DAPI), 1,4-diazabicyclo[2.2.2]octane (DABCO), and bovine serum albumin were purchased from Sigma-Aldrich. Rabbit polyclonal anti–type IV collagen (Pasteur Institute) was provided by the Laboratory on Thymus Research, Instituto Oswaldo Cruz, Rio de Janeiro. Secondary AlexaFluor 488 goat anti-rabbit IgG was purchased from Molecular Probes (Invitrogen). For cardiomyocyte isolation and cultivation, trypsin was obtained from Difco Laboratories, type II collagenase from Worthington Biochemical, and fetal bovine serum (FBS), 1-glutamine, penicillin, streptomycin, and Dulbecco’s modified Eagle medium from Sigma-Aldrich.

**Primary cardiac-cell cultures.** Swiss Webster mice were obtained from CECA-FIOCRUZ. They were bred and manipulated according to recommendations approved by the FIOCRUZ Ethical Committee in Animal Use (CEUA-FIOCRUZ protocol 232/04). The hearts of 18-day-old mouse embryos were subjected to enzymatic dissociation using 0.05% trypsin and 0.01% collagenase in PBS (pH 7.2) at 37°C, as described else-

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**Figure 2.** Hypertrophy and remodeling of cardiac microtissue, induced by *Trypanosoma cruzi* infection. A and B, Three-dimensional (3D) confocal microscopy of spheroid stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) after 13 days of culture. The spheroid shown in panel B was analyzed 6 days (144 h postinfection [144hpi]) after infection and showed augmented volume, compared with that in a noninfected control (A). C and D, Optical sections, as shown by confocal microscopy, of whole-mount spheroids, showing *T. cruzi*-induced hypertrophy in 3D cultured cardiomyocytes. DAPI (blue) was used to stain host-cell and parasite (denoted by arrows in panel D) nuclei. Evans staining (red) allowed analysis of cell morphology. D, At 144hpi, cardiomyocytes displayed an increase in cellular area (white-bordered areas). E, Histogram showing that spheroid volume was increased at 144hpi, compared with that in noninfected control spheroids. Volume measurements performed by use of LSM Image Browser software revealed a 5.9-fold increase in the volume of infected spheroids (P < .05, by analysis of variance [ANOVA]). F, Histogram showing that the infection induced a 4.8-fold increase in the cellular area of the cardiomyocytes (P < .05, by ANOVA). G, Histogram showing the number of nuclei in the spheres, revealing a 50% reduction in cell density (P < .05, by ANOVA).
where [10]. To allow formation of cardiac spheroids (3D micro-
tissues), 25 × 10^3 cardiomyocytes were plated in agarose-
coated 96-U-well plastic plates. Cells were maintained at 37°C in
a 5% CO2 atmosphere in Dulbecco’s modified Eagle medium
supplemented with 10% FBS, 1 mmol/L CaCl2, 1 mmol/L
L-glutamine, 2% chick-embryo extract, 1000 U/mL penicillin,
and 50 µg/mL streptomycin.

**T. cruzi infection of cardiac microtissue.** Trypomastigote
forms of *T. cruzi Y* strain (MHOM/BR/1950/Y) were obtained
from the supernatant of previously infected Vero cells grown in
RPMI medium (CultiLab) supplemented with 10% FBS. After 7
days of culture, cardiac spheroids were infected at a 20:1 parasite:
cardiomyocyte ratio. At specific times, infected and noninfected
cardiac microtissues were washed 3 times in PBS and were fixed
for use in different microscopy assays.

**Transmission electron microscopy (TEM) and scanning
electron microscopy (SEM).** Microtissue samples were fixed
for 60 min at 4°C with 2.5% glutaraldehyde in 0.1 mol/L cac-
dylate buffer (pH 7.2). After postfixation for 30 min with 1%
OsO4/0.8% potassium ferricyanide/2.5 mmol/L CaCl2 in the
same buffer, the samples were dehydrated in an acetone series
(30%–100%). For TEM, the material was embedded in Poly-
Bed812 (Polysciences) resin. Ultrathin sections were stained
with uranyl acetate and lead citrate and were examined by use of
a Zeiss EM10C transmission electron microscope; for SEM, the
spheroids were critical-point dried with CO2, were mounted on
aluminum stubs, and were coated with a 20-nm-thick layer of
gold. The samples were examined by use of a Zeiss DSM-640
scanning electron microscope.

**Immunofluorescence and confocal microscopy.** Cardiac
spheroids were fixed in 4% paraformaldehyde (PFA) for 1 h at
4°C, were embedded in Tissue-Tek ornithine carbamoyltrans-
ferase compound (Sakura Finetechnical, Japan), were stored at
−80°C, and then were sliced via cryomicrotomy. For whole-
mount analyses, spheroids were fixed for 1 h with 4% PFA and
were permeabilized overnight with 0.5% Triton X-100. All
washes were performed with Triton X-100, in the same concen-
tration. After 1 h of blockage in PBS/3% bovine serum albumin,
the slices and whole-mount spheroids were incubated overnight
at 4°C with primary antibody against fibronectin, collagen IV,
or laminin. The samples were then washed 3 times with PBS and
were incubated, at either 37°C for 2 h (cryosections) or overnight
at 4°C (whole-mount spheroids), with a secondary AlexaFluor
488 antibody. Nuclear DNA was stained with DAPI diluted in
PBS. Whole spheroids were resuspended in DABCO antifading
agent and were placed in confocal dishes (Mattek); cryosections
were mounted by use of cover slips. Analyses were performed by
use of a Zeiss 510Meta laser scanning confocal microscope.

**Analysis of hypertrophy of cardiac microtissue.** The size of
spheroids and cardiomyocytes was measured at 144 h (6 days)
postinfection, when most cells were already infected with *T.
cruzi*. Microtissues were washed in PBS, fixed for 1 h at 4°C in 4%
PFA, and maintained in 0.5% Triton X-100 overnight at 4°C.
They were then washed in PBS, and DNA staining using DAPI
was performed. The spheroids were then stained for 1 min with
0.1% Evans blue, resuspended in DABCO, placed in Mattek con-
focal dishes, and observed via confocal microscopy.

**Quantifications and statistical analysis.** The percentage of
stained area of ECM compounds in the cardiac spheroids was
measured by use of Image-Pro-Plus software. The number of

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*Figure 3. Trypanosoma cruzi-induced fibrosis in cardiac microtissues.*

3D confocal microscopy revealed that expression and immunoreactivity of
fibronectin in microtissues was increased at 144 h postinfection (144hpi)
(B), compared with that in control microtissues (A). Images were acquired
after whole-mount immunostaining of cardiac spheroids.
nuclei and the area of myocytes, the volume and diameter of spheroids, and the number of cells per diameter of spheroids were estimated by use of LSM Image Browser software (Zeiss). Ten confocal slices from the top to the middle of whole-mount cardiac microtissues were obtained. Statistical analyses were performed by an analysis-of-variance test, with $P < .05$ as the level of significance. The data are representative of 3 independent experiments, with 10 spheroids per experiment.

**Figure 4.** *Trypanosoma cruzi* infection–induced increase in expression of fibronectin in cardiac spheroids. Cryosections of control (left-side panels) and infected (right-side panels) microtissues were stained either with 4',6-diamidino-2-phenylindole dihydrochloride (A and B) or with rabbit anti-fibronectin primary antibody and AlexaFluor 488 anti-rabbit secondary antibody (C and D). Merged images of panels A and C (E) and of panels B and D (F) are also shown. Confocal analysis revealed alterations in the distribution of and increased fibronectin staining in infected spheroids (D and F). Whereas the staining in control microtissues was present mainly at the cell periphery (C and E), that in infected spheroids was diffused (**) throughout the microtissue (D and F). In regions with amastigote nests (arrows), there was a reduction in fibronectin staining, as shown in panels B, D, and F. Inserts in panels E and F show a general view of fibronectin staining and DNA staining in spheroids.
RESULTS

Effects of *T. cruzi* infection on cardiac microtissues. After 7 days of culture, cardiac microtissues were infected with *T. cruzi* try- 
opomastigotes. The complete parasite cycle in the spheroids could be 
followed via electron microscopy. The surface of the microtissue 
was visualized by SEM (figure 1A–1C), which revealed round cells 
over a continuous surface (figure 1A), as well as cells with mem-

Figure 5. Expression of collagen IV in *Trypanosoma cruzi*-infected cardiac microtissues. Cryosections of control (left-side panels) and infected (right-side panels) microtissues were stained with either 4',6-diamidino-2-phenylindole dihydrochloride (A and B) or rabbit anti–collagen IV primary antibody and AlexaFluor 488 anti-rabbit secondary antibody (C and D). Merged images of panels A and C (E) and of panels B and D (F) are also shown. Confocal analysis revealed an increased staining of this protein in infected spheroids. In regions with amastigote nests (denoted by arrows in panels B, D, and F), there was intense collagen reactivity. Inserts in panels E and F show a general view of spheroids stained for collagen IV and for DNA.
brane projections (figure 1A and 1B). At 96 h postinfection, trypanomastigotes were released from the host cells, some of which had signs of damage, such as exposed intracellular structures (figure 1C), that were not observed in control microtissues (figure 1B). Furthermore, trypomastigotes could be observed in the supernatant after that time (data not shown).

Figure 6. Drastic alteration of laminin distribution and deposition in Trypanosoma cruzi–infected cardiac spheroids. Cryosections of control (left-side panels) and infected (right-side panels) spheroids were stained with either 4',6-diamidino-2-phenylindole dihydrochloride (A and B) or rabbit anti-laminin primary antibody and Alexa Fluor 488 anti-rabbit secondary antibody (C and D). Laminin was present in the cell periphery, resulting in a meshwork pattern (denoted by asterisks in panel C). Confocal analysis revealed both an increase in laminin staining and total disorganization of the meshwork architecture of this protein in infected spheroids (B, D, and F). Strong staining was present in regions with amastigote nests (denoted by arrows in panels B, D, and F). Inserts in panels E and F show a general view of spheroids stained for laminin and for DNA.
TEM showed cardiomyocytes with ultrastructural characteristics of the in vivo cardiac tissue, such as myofibrils, abundant mitochondria, sarcoplasmic reticulum profiles, and intercellular junctions (figure 1D). At 24 h postinfection, recently interiorized trypomastigotes were observed inside parasitophorous vacuoles, showing the typical basket-shaped kinetoplast (figure 1E). At 48–72 h postinfection, amastigote forms were detected proliferating in the host-cell cytoplasm, displaying typical basket-shaped kinetoplasts (figure 1F). At 96 h postinfection, trypomastigotes were released from the myocytes (figure 1C) and reininfected the microtissue, resulting in infection of almost all cells at 144 h postinfection, as demonstrated by DNA staining with DAPI (figure 2D).

**T. cruzi infection and hypertrophy of cardiac spheroids.** Observation of multiple optical sections via confocal microscopy revealed that cardiac spheroids containing ~25,000 cells were 295 ± 12 μm in diameter and 16 ± 1 cells wide. At 144 h postinfection, the infected cardiac spheroids showed a 5.9-fold increase in volume (figure 2B and 2E), compared with noninfected ones (figure 2A). To verify whether this phenomenon was related to cell proliferation, nuclei in microtissues were stained with DAPI and then were quantified. Surprisingly, infected microtissues displayed 50% fewer nuclei than did control microtissues (figure 2G). Staining of the spheroids with Evans blue allowed quantification of the cellular area (figures 2C and 2D) and showed that the infection caused hypertrophy of the cardiomyocytes, as demonstrated by a 4.8-fold increase (P < .05) in the cellular area (figure 2F).

**Cryosection analysis of T. cruzi–induced fibrosis of cardiac microtissues.** Together with the reduction in cellularity, an increase in ECM deposition, as shown by immunostaining of ECM components, was observed by confocal microscopy using 3D reconstruction, both in infected cardiac microtissue (figure 3) and in cryosections (figures 4–6). Noninfected cardiac spheroids showed fibronectin staining mainly of the microtissue surface (figure 4C and 4E), corresponding to 6% of the total cellular area (figure 7A), whereas infected spheroids showed intense staining of both the surface and the interior, corresponding to 12% of the total cellular area (figure 7A). In infected cells, there was faint fibronectin staining around the amastigotes. However, the staining in noninfected cells in infected microtissues was more intense than that in control spheroids (figure 4D and 4F). Staining of collagen IV was increased in infected microtissues (figure 5D and 5F), corresponding to 40% of the total cellular area, versus 10% of that in control spheroids (figure 7B). Staining of collagen IV also was observed amid T. cruzi amastigotes in infected cells, as well as around infected and noninfected cells (figure 5D and 5F).

During the infection, laminin staining showed a remarkable alteration in both distribution pattern and immunoreactivity. In noninfected microtissues, laminin staining was organized as a meshwork (denoted by asterisks in figure 6C) that was present between cells, mainly at the cell periphery (figure 6C and 6E), and corresponded to 15% of the total cellular area (figure 7C); on the other hand, in infected spheroids (figure 6D and 6F), intense laminin staining was present not only at the cell periphery but also in the cytoplasm of most noninfected cells and corresponded to 40% of the total cellular area (figure 7C). In infected cells, staining was located mainly around parasite nests (figures 6D and 6F). In the expression of all ECM components analyzed, significant differences (P < .05) between noninfected control spheroids and infected spheroids were observed (figure 7).
DISCUSSION

Functional cardiac spheroids have been developed for in vitro studies by bioengineering. They are compact cellular aggregates that are characterized by spontaneous contractility, differentiated cardiomyocytes, production of growth factors and ECM components organized in a 3D network (L.R.G., D.A., M.J.S., M.I.D.R., R.B., and M.d.N.L.d.M., unpublished data). During coculture with endothelial cells, the vascular endothelial growth factor produced by cardiac microtissues is able to modulate both migration of these cells and angiogenesis [23; L.R.G., D.A., M.J.S., M.I.D.R., R.B., and M.d.N.L.d.M., unpublished data].

The aim of the present study was to reproduce, in vitro, conditions related to chagasic cardiomyopathy, by using a 3D cultivation system to infect cardiomyocytes (cardiac spheroids) with T. cruzi. In microtissues produced in vitro, cells are connected to their neighbors and to the ECM, forming a complex 3D environment that is not reproduced in 2D (monolayer) systems [21, 24]. The 3D organization results in patterns of gene expression and other biological activities that mirror more closely what happens in living organisms [25, 26].

Cardiac spheroids were obtained and then were infected with T. cruzi trypomastigotes for the first time. Ultrastructural and confocal analyzes demonstrated that the parasites were able to invade the host cells, to differentiate into multiplicative amastigote forms, to complete the intracyclic cycle, and to reinfect new cardiomyocytes, resulting, at 144 h postinfection, in infection of almost all host cells. In summary, we were able to observe the 5 stages of the intracellular cycle of T. cruzi—namely, invasion, trypomastigote-to-amastigote differentiation, amastigote proliferation, amastigote-to-trypomastigote differentiation, and escape of trypomastigotes from the host cells [27].

In vivo, chagasic cardiomegaly results from multifocal inflammatory mononuclear infiltrates, myocytolytic necrosis, myocardial hypertrophy, interstitial fibrosis [28, 29], and an increase in the dimension of the right ventricular chamber, during both phases (i.e., acute and chronic) of infection [30]. However, results reported elsewhere had shown that, independent of inflammatory mononuclear cells, infection of cardiomyocyte monolayers by T. cruzi induced a 2-fold increase in the cellular area of the host cells [5, 31]. In the present study, 3D culture showed that, after T. cruzi infection, there were alterations not only in the cellular area but also in the total volume of cardiac microtissue. Surprisingly, we observed a significant reduction in the number of nuclei, suggesting cell death, despite the noticeable increase in volume observed in the spheres. The results of the present study suggest that the increase in volume was due not only to cell hypertrophy but also to the deposition of ECM components: a large fibrosis was observed, caused by an increased expression of fibronectin, collagen IV, and laminin; furthermore, these proteins had an altered distribution in infected spheroids. The results of the present study mimic aspects of acute in vivo infection with T. cruzi, showing destruction of the myocardial fibers, intense repairing fibrosis, and, consequently, CR.

Despite the important role that inflammatory cells play in fibrosis genesis in chagasic cardiomyopathy [3], recent in vitro studies using monolayers have suggested that host cells play a direct role in fibrosis during T. cruzi infection. In cultured fibroblasts, parasite infection increased the production of ECM proteins [32]; on the other hand, it has been shown that T. cruzi inhibits the production of TGFβ-dependent growth factor in connective tissue, resulting in reduction in the production of both fibronectin and collagen I [33]. Evidence provided by monolayered cardiomyocytes has shown mechanisms concerning T. cruzi–mediated response in these cells, mechanisms involving both ECM production and cardiomyocyte hypertrophy, by modulation of TGFβ [6] and IL-1β [31] respectively. Again, controversial results have been reported. Despite the fact that in vitro infection with T. cruzi induces increased production of TGFβ in cardiomyocytes [6] and (2) that TGFβ in vivo is involved in fibrosis and myocardiacopathy in Chagas disease [34, 35], the monolayers of cardiomyocytes did not confirm a previous study’s report that T. cruzi in vivo induces increased expression of fibronectin [18]. This failure can be related to the limitations of the 2D culture systems in monolayers. In 2D culture systems, the ECM components are secreted and reach the liquid culture medium, impairing the assembly of fibronectin into fibrils [36]. On the other hand, the results of the present study clearly show that fibronectin, collagen IV, and laminin have an increased immunoreactivity in T. cruzi–infected cardiospheres. In a 3D culture, it is easier for these proteins to assemble properly into fibrils and to interact with other ECM components and/or molecules of the cell surface, thus staying confined within the 3D network.

In summary, the results of the present study show that (1) T. cruzi is able to infect and to complete its intracyclic cycle in cardiac microtissue produced in vitro and (2) use of a 3D culture system allows observation of hypertrophy, fibrosis, and remodeling in cardiac spheroids after T. cruzi infection, thus reproducing aspects of heart alteration that are observed during chagasic cardiomyopathy. We believe that the 3D cardiomyocyte-culture system presented here can contribute to future studies of the processes involved in cardiac-tissue remodeling during T. cruzi infection. In addition, cardiac spheroids can be used to study other mechanisms involved in cardiac hypertrophy and fibrosis in distinct cardiac pathological conditions.

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References


