Activation of Transcription Factors AP-1 and NF-κB in Murine Chagasic Myocarditis

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The myocardium of CD1 mice was examined for the activation of signal transduction pathways leading to cardiac inflammation and subsequent remodeling during Trypanosoma cruzi infection (Brazil strain). The activity of three pathways of the mitogen-activated protein kinases (MAPKs) was determined. Immunoblotting revealed a persistent elevation of phosphorylated (activated) extracellular-signal-regulated kinase (ERK), which regulates cell proliferation. During infection there was a transient activation of p38 MAPK but no activation of Jun N-terminal kinase. Early targets of activated ERK, c-Jun and c-Fos, were elevated during infection, as demonstrated by semiquantitative reverse transcription-PCR. Immunostaining revealed that the endothelium and the interstitial cells were most intensely stained with antibodies to c-Jun and c-Fos. Soon after infection, AP-1 and NF-κB DNA binding activity was increased. Protein levels of cyclin D1, the downstream target of ERK and NF-κB, were induced during acute infection. Immunostaining demonstrated increased expression of cyclin D1 in the vascular and endocardial endothelium, inflammatory cells, and the interstitial areas. Increased expression of the cyclin D1-specific phosphorylated retinoblastoma protein (Ser780) was also evident. Immunoblotting and immunostaining also demonstrated increased expression of proliferating cellular nuclear antigen that was predominantly present in the inflammatory cells, interstitial areas (i.e., fibroblasts), and endothelium. These data demonstrate that T. cruzi infection results in activation of the ERK-AP-1 pathway and NF-κB. Cyclin D1 expression was also increased. These observations provide a molecular basis for the activation of pathways involved in cardiac remodeling in chagasic cardiomyopathy.

Infection with the protozoan hemoflagellate parasite Trypanosoma cruzi causes Chagas’ disease. The important manifestations of Chagas’ disease include acute myocarditis and chronic cardiomyopathy (37, 57). Chagas’ disease continues to be a serious health problem in Mexico and Central and South America and has recently emerged as an opportunistic infection in the setting of AIDS (43).

T. cruzi infection is lifelong, and chagasic heart disease represents a unique interplay of ischemic and inflammatory changes, resulting in cardiac myocyte hypertrophy (2), cardiac remodeling, and the eventual development of chronic cardiomyopathy. Acute chagasic myocarditis plays an important role in the development of chronic cardiomyopathy and is characterized by intense inflammation, myonecrosis, myocytolysis, vasculitis, and numerous parasite pseudocysts. These pathological changes are accompanied by the increased expression of myocardial cytokines, chemokines, nitric oxide synthase, endothelin-1 (ET-1), and kinins (10, 18, 19, 38, 45, 56, 57, 60). Chagasic heart disease is also accompanied by vasculopathy (37, 41), manifested by microvascular spasm and decreased blood flow (37, 57, 58). Similarly, in the other examples of myocardial injury activation of several signaling pathways involving cytokines, chemokines, NF-κB, vascular adhesion molecules, transforming growth factor beta, and ET-1 and the mitogen-activated protein kinases (MAPKs) is observed (4, 9, 23, 24, 30–32, 42, 48, 50, 55, 59).

Eukaryotic cell division has been divided into distinct phases, originating from observations of periods of distinct biological activity. The orderly progression of cells through the phases of the cell cycle is governed by the sequential assembly and activation of holoenzyme complexes, comprised of a regulatory subunit (cyclin) and a catalytic subunit (cyclin-dependent kinase [Cdk]), both of which are distinct for each phase (5, 35, 39). The MAPK pathways, ET-1, and cell cycle-regulatory proteins, including cyclin D1, participate in the regulation of cell proliferation and cardiac remodeling (4, 7, 24, 27, 28, 37, 42, 54). Importantly, cyclin D1, a regulator of cellular proliferation, is itself regulated by extracellular-signal-regulated kinase (ERK), a component of the MAPK pathway and ET-1 (35, 52). The cell cycle-regulatory protein cyclin D1 is an important mediator of G1 phase progression and a downstream target of multiple proliferative signaling pathways, including MAPKs, NF-κB, and activating transcription factor 1 (AP-1) (5, 35, 39, 47).

We found that myocardial injury following T. cruzi infection resulted in increased expression of those proteins known to be associated with cellular proliferation, such as proliferating cell...
nuclear antigen (PCNA). Since the MAPK pathways have been implicated in cellular proliferation as a result of myocardial injury (48), we undertook an investigation of this pathway in chagasic myocarditis. T. cruzi infection was found to activate ERK and the transcription factors AP-1 and NF-κB as well as the downstream target cyclin D1. These data suggest that activation or induction of these signaling pathways in T. cruzi-induced cardiovascular disease likely contributes to the observed cardiovascular remodeling as assessed by histopathology and analysis of myocardial structure and function (8, 19). Furthermore, these studies into the mechanisms that control the proliferation of cells of the cardiovascular system during T. cruzi infection are important in advancing our understanding of the pathogenesis of chagasic heart disease and in providing possible targets for adjunctive therapy.

**MATERIALS AND METHODS**

**Infection of mice.** Eight- to 10-week-old male CD1 mice (Jackson Laboratories, Bar Harbor, Maine) were infected with 5 × 10⁴ trypomastigotes of the Brazil strain of T. cruzi. Hearts were obtained for histology, immunostaining, and protein and RNA isolation.

**Immunoblotting.** Homogenates were obtained from mouse hearts as previously described (20). Approximately 30 to 50 μg of homogenate was dissolved in sample buffer, boiled for 5 min, and separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12% PAGE). Samples were then transferred onto nitrocellulose membranes (0.2 μM) by electroblotting. Analysis of phosphorylated (activated) ERK, Jun N-terminal kinase (JNK), p38 MAPK, and retinoblastoma protein (pRb) was performed with antibodies obtained from Cell Signaling (Beverly, Mass.). Antibodies to ERK and p38 MAPK were polyclonal, while the antibody to JNK was monoclonal. Analysis of PCNA was performed with a monoclonal antibody obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). The abundance of cyclin D1 protein was determined with a monoclonal cyclin D1 antibody, AB3 (polyclonal) or DCS-6 (monoclonal) (Neo-Markers, Fremont, Calif.). Filters were probed with primary antibodies at a dilution of 1:500, and the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz) was used at a dilution of 1:5,000. Signals of bound antibodies were visualized with Western blotting protocols (ECL; Amersham Life Science). Coomassie blue staining was used to determine protein integrity, and a GDP-disassociated inhibitor antibody, a gift of P. Scherer, was used to assess equal loading efficiency.

**RT-PCR studies.** Total RNA was isolated from mouse hearts with Trizol reagent according to the protocol of the manufacturer (Gibco-BRL, Grand Island, NY).
speci
primary antibodies. In all cases this prevented immunostaining, con
Medicine. Control slides for all reactions were done with the omission of the
were imaged at the Analytical Imaging Facility at the Albert Einstein College of
with a standard coverslip, and the edges were sealed with nail polish. The slides
1:150 dilution. The samples were again washed thrice for 10 min each in phos-
–
3% bovine serum albumin according
frozen myocardium was broken into small pieces in the presence of liquid
extracts were prepared as described by Chandrasekar et al. (9). Brie-
y, the
resed in a 1.6% agarose gel containing ethidium bromide.
Electrophoretic mobility shift assays for AP-1 and NF-κB. Cardiac protein
extracts were prepared as described by Chandrasekar et al. (9). Briefly, the
frozen myocardium was broken into small pieces in the presence of liquid
and protein extracts were prepared by homogenizing (Polytron; 4°C;
8,000 rpm) the tissue in a buffer containing 20 mM HEPS (pH 7.8), 300 mM
NaCl, 0.4 mM EDTA, 0.5 mM dithiothreitol, 25% glycerol, and 0.5 mM phe-
nethylsulfonyl fluoride and centrifuged at 14,000 rpm for 10 min at 4°C. Protein extract concentration was determined with the Bio-Rad assay. The ex-
tracts were then frozen on dry ice and stored at −80°C. An aliquot of 20 μg of cardiac
protein extracts from infected mice and uninfected mice were checked for
[32P]AP-1 and [32P]NF-κB consensus sequence oligonucleotide (AP-1: 5′-GCC
TTG ATG AGT CAG GAA-3′; NF-κB: 5′-AGT TGA GGC TAT CTC
CCC AGG C-3′) from Promega, Madison, Wis.) binding by electrophoretic
mobility shift assay as previously described (18). A 50-fold molar excess of unlabelled oligonucleotides (AP-1, NF-κB, and SP-1) was used for competitive
assays to determine the specificity. In addition, specific antibodies for c-Fos and
c-Jun as well as p50 and p65 (Santa Cruz Biotechnology, Santa Cruz, Calif.) were used for a supershift assay to identify the proteins involved in the complexes, as
previously described (18).
Immunostaining. These studies were performed with polyclonal antibodies to
c-Fos, c-Jun, and PCNA (Santa Cruz Biotechnology), cyclin D1 (Cell Signaling),
and the NF-κB components p50 and p65 (polyclonal antibodies obtained from
Santa Cruz Biotechnology). Paraffin-embedded sections were used for these antibodies. For studies on phospho-ERK, hearts were immediately removed,
embedded in optimal cutting temperature compound, and the endothe-
lium were the most intensely stained in samples obtained from the
myocardium of infected mice (Fig. 1D and 1E). Such stain-
ing was evident at 15, 30, 60, and 150 days postinfection. Im-
munoblotting done at 60 days postinfection detected an in-
creased expression in the myocardium (Fig. 2A).
Immunoblotting for phosphorylated ERK, JNK, and p38
MAPK in the myocardium of infected mice. Immunoblotting
with an anti-PCNA antibody revealed that the inflamma-
tory cells, the interstitial areas (fibroblasts), and the endo-
terolysis of CD1 mice days 1 to 30 postinfection. (A) PCNA expres-
sion was induced 60
Proliferating cell nuclear antigen expression. Immunostain-
ing with an anti-PCNA antibody revealed that the inflamma-
tory cells, the interstitial areas (fibroblasts), and the endo-
terolysis of CD1 mice days 1 to 30 postinfection. (A) PCNA expres-
sion was induced 60

RESULTS
Parasitology and pathology. The mortality of Brazil strain-
infected CD1 mice was 60% by day 35 postinfection. These
data are consistent with our previously published studies on mortality with this strain of T. cruzi in CD1 mice (8). His-
topathological examination of the myocardium of Brazil-in-
fected CD1 mice revealed myonecrosis, inflammation, vascu-
litis, and multiple parasite pseudocysts (Fig. 1A, 1B, 1C, and
1G).
FIG. 4. Phospho-ERK expression. Representative myocardial sections obtained from uninfected and T. cruzi-infected mice 15 days postinfection. The upper panels represent phase contrast microscopy of representative interstitial areas (arrows) composed of fibroblasts and the endothelium. Immunostaining of frozen myocardial sections was performed with antibodies to phosphorylated ERK (red stain). Anticaveolin-3 antibody was used as a marker of cardiac myocytes (green stain). There was no phosphorylated ERK staining in the uninfected section. The double-stained sections did not demonstrate phosphorylated ERK expression in cardiac myocytes.
phorylated p38 MAPK at day 15 postinfection, while the level of phosphorylated JNK was unchanged (Fig. 3).

**Immunostaining for phosphorylated ERK.** To determine the origin of the activated ERK in the myocardium, we performed immunostaining of frozen myocardial sections obtained from uninfected and infected mice (day 15 postinfection). We used antibodies to phosphorylated ERK and caveolin-3. Anti-caveolin-3 antibody was used as a marker of cardiac myocytes (Fig. 4). There was no phosphorylated ERK staining in the uninfected section. In addition, the double-stained sections did not demonstrate activated ERK in cardiac myocytes but rather in the interstitial areas containing fibroblasts.

**Semi-quantitative RT-PCR for c-fos and c-jun in myocardium of infected mice.** c-fos and c-jun are downstream targets of ERK. Therefore, we performed RT-PCR with specific c-fos and c-jun primers to examine the expression of these early-response genes in response to T. cruzi infection. The results were analyzed by densitometry, and the ratio of the optical density of c-fos to GAPDH and of c-jun to GAPDH was used to demonstrate gene expression. There was no expression in uninfected control mice. There was a significant increase in abundance in infected mice from days 1 to 15 postinfection compared with uninfected mice (Fig. 5). At days 30 and 60 postinfection, the expression of both genes was still detected by RT-PCR (data not shown).

**Immunostaining for c-Jun and c-Fos.** These studies revealed staining of c-Fos and c-Jun in the myocardium, endothelium, endocardium, and capillaries. However, staining was more intense in the vascular endothelium and endocardial endothelium obtained from infected mice (Fig. 11). c-Fos and c-Jun were detected in the myocardium at early (day 14 postinfection) and late (day 120 postinfection; data not shown) time points.

**AP-1 assays.** The transcription factor AP-1 is a downstream target of ERK. Therefore, we performed studies to examine consensus sequence oligonucleotide binding specificity and determine the proteins involved in the binding complexes. Cardiac protein extracts from infected mice at 15 days postinfection and age-matched uninfected mice were analyzed for \[^{32}P\]AP-1 consensus sequence oligonucleotide binding by electrophoretic mobility shift assay (Fig. 6B). There was a significant increase in DNA binding activity in infected versus uninfected samples (Fig. 6B, lanes 1 and 2). In lane 3, pretreatment with the 50-fold excess of unlabeled AP-1 but not SP1 (Fig. 6B, lane 4) consensus oligonucleotide completely abolished the signal. Supershift assay with either anti-c-Jun (Fig. 6B, lane 5) or anti-c-Fos (Fig. 6B, lane 6) antibodies decreased the intensity of the signal. Supershift assays with both anti-c-Jun and anti-c-Fos antibodies almost completely abolished the signal (Fig. 6B, lane 7). However, supershift assay with nonimmunized rabbit serum failed to suppress the signal, indicating the effects of c-Jun and c-Fos antibodies interaction with the protein extracts were specific (Fig. 6B, lane 8).

We performed a time course of AP-1 DNA binding activity in the hearts of infected mice. In Fig. 6A, electrophoretic mobility shift assay detected increased AP-1 DNA binding activity from day 2 to day 14 postinfection as a result of infection with T. cruzi. The increased AP-1 DNA binding activity was also found at days 30 and 60 postinfection (data not shown). These studies indicate that AP-1 is activated as a result of infection.

**NF-κB assays.** NF-κB is a transcription factor that contributes to the induction of cytokines and cyclin D1. We examined \[^{32}P\]NF-κB consensus sequence oligonucleotide binding activity (Fig. 7). There was a low background signal in uninfected mice (Fig. 7, lane 1). NF-κB DNA binding was increased in the myocardium of infected mice (Fig. 7, lane 2). Competition assay with unlabeled oligonucleotides (NF-κB and SP-1) indicated that the binding was specific to NF-κB (Fig. 7, lanes 3 and 4). Supershift assays demonstrated that the signal contained p50 and p65 subunits (Fig. 7, lanes 5, 6, and 7). Immunohistochemistry employing antibodies to p50 and p65 showed more intense staining in the vasculature (Fig. 1H).

**Cyclin D1 expression.** Cyclin D1 is an important downstream target of ERK and NF-κB. Western blot revealed that the expression of cyclin D1 was increased by the first day of infection and persisted through day 60 postinfection (Fig. 2B). Immunohistochemistry revealed the most intense staining to be in the vascular and the interstitial areas of infected myocardium (Fig. 1F).

**Retinoblastoma protein.** Immunoblotting revealed that the phosphorylation of cyclin D1-specific pRB (Ser780) was increased from day 1 to day 60 postinfection (Fig. 2C). There was no increase in pRb phosphorylation with Ser380 antibody (non-cyclin D1 specific; data not shown). In addition, expression of total pRB was unchanged. These observations underscore the specificity of the cyclin D1 contribution to cardiac remodeling (8, 19).

**DISCUSSION**

During acute infection, T. cruzi gains access to the cardiac myocytes by first invading endothelial cells, the interstitial areas of the vascular wall, and the myocardium. Thus, damage to these cells and the extracellular matrix occurs as a result of the invasion process, parasite products, and the inflammatory re-
sponse. Subsequently, remodeling occurs, resulting in structural changes associated with inflammation, necrosis, cardiac myocyte hypertrophy, fibrosis, and ventricular dilation as well as aneurysm formation (57). These phenomena have been demonstrated in human disease and in animal models. However, the mechanisms of cellular proliferation and subsequent cardiac remodeling in Chagas’ disease has not been explored in detail.

In the present report as well as previously published studies, we observed evidence of cardiovascular remodeling. This was demonstrated by histopathology, echocardiography, and cardiac magnetic resonance imaging (8, 19). In this paper, immunohistochemistry with anti-PCNA antibody demonstrated evidence of cellular proliferation in the myocardium as early as 15 days postinfection, which did not appear to involve cardiac myocytes. However, Arnaiz et al. (2) recently demonstrated PCNA-positive staining in the inflammatory areas of the myocardium of T. cruzi-infected rats, including cardiac myocytes. Since the MAPK pathway has been shown to be an important regulator of cellular proliferation and remodeling in animal models of myocardial injury (4, 30, 38, 46, 52), we investigated the activation of this pathway in the myocardium of T. cruzi-infected mice. Of the three major MAPKs in mammalian cells (ERK, JNK, and p38 MAPK), ERK and JNK contributed to the activation of AP-1. By immunoblotting, we demonstrated induction of ERK phosphorylation, which was persistent, in the myocardium of infected mice. Immunostaining suggested that the source of the phosphorylated ERK was the interstitial areas of the infected myocardium. These areas contained fibroblasts and inflammatory cells. Similar areas of uninfected myocardium did not reveal staining. Cardiac myocytes were shown not to be the major source of phosphorylated ERK in infected mice.

The induction of ERK in fibroblasts likely causes induction of factors such as ET-1 and transforming growth factor beta, which have been shown to be important in cardiac myocyte...
Among the downstream targets of ERK is the family of AP-1 transcription factors composed of c-Jun and c-Fos proteins. AP-1 has been shown to induce the synthesis of ET-1 (30) and transforming growth factor beta (16, 51). Our present study clearly demonstrates that T. cruzi infection activates the ERK-AP-1 pathway, which has been shown to induce the synthesis of ET-1 (30).

Coronary angioplasty and subsequent restenosis are other models of vascular injury that have been investigated extensively. The injury to the vessel results in smooth muscle cell proliferation, migration, and the production of extracellular matrix. Various therapies have been investigated to reduce neointimal formation in vessels treated with balloon angioplasty. For example, the use of sirolimus-eluting stents has shown promise in reducing restenosis. However, the mechanism by which these therapies work is not fully understood. Further research is needed to fully elucidate the role of transcription factors such as AP-1 in the development of restenosis and the identification of new therapeutic targets.
antisense oligonucleotides directed against cyclin-dependent kinase 2 and/or cell division cycle 2 and PCNA inhibited smooth muscle cell proliferation (34, 53, 63). Likewise, localized arterial injection of a nonphosphorylatable form of a dominant negative protein gene product (pRB) at the time of angioplasty blocked cellular proliferation (11).

Chagas heart disease is a result of ischemia and inflammatory injury. Although alterations in myocardial signaling have been explored extensively in other forms of cardiovascular injury, such as that following myocardial infarction and balloon angioplasty, these events have not been fully investigated in chagasic heart disease. Our data are consistent with the idea that activation of ERK, AP-1, and NF-κB and downstream targets such as ET-1 and cyclin D1 contributes to cardiogenic remodeling following injury due to T. cruzi infection. The possibility of targeting components of the MAPK or the cell cycle pathways is now gaining acceptance as a therapeutic modality for cardiovascular disease, and the data in this paper suggest that this type of adjunctive therapy may be also useful in Chagas’ disease (1, 6, 21, 27, 34, 49).

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