Targeted myocardial transgenic expression of HIV Tat causes cardiomyopathy and mitochondrial damage

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HIV Tat serves as a transactivator that stimulates transcription and is required for efficient HIV replication (reviewed in Ref. 17). Tat can activate heterologous promoters and mediate activities of cellular functions. Extracellular Tat promotes growth of spindle cells derived from Kaposi’s sarcoma and normal vascular cells (2, 15). Tat contributes to the activation of endothelial cells and the expression of endothelial cell adhesion molecules (12, 20).

HIV has been demonstrated in cardiac myocytes in AIDS (4, 19, 32). However, the cardiac effects of Tat are poorly understood. Tat may impact on drug toxicity and cause oxidative damage in organs and has been shown to decrease glutathione (GSH) content in livers of transgenic mice (TG) (9). In light of these effects, our working hypothesis states that Tat impacts directly on myocardial cellular function in AIDS patients and contributes to AIDS CM. Experiments in the present study explored the effect of Tat on the structure and function of the cardiac myocyte and of the heart.

Previously, HIV Tat was ubiquitously and nonspecifically expressed in TG with various promoters (e.g., β-actin promoter) (7–10, 18, 25, 35, 40). However, those TG models examined Tat effects in noncardiac tissues, lacked tissue targeting, exhibited no Tat myocardial expression, or exhibited Tat expression in multiple tissues without examining cardiac effects. Accordingly, cardiac effects (if any) from ubiquitous TG expression of Tat could result from systemic, local, or combined effects of Tat expression.

Experiments here targeted expression of Tat to cardiac myocytes and focused on changes in myocytic and cardiac structure and function that resulted from Tat expression. An established TG strategy (33, 37) was used to specifically target HIV Tat to murine cardiac myocytes. Selective expression of HIV Tat in the myo-
cardium increased left ventricular (LV) mass, decreased ventricular fractional shortening (FS), caused mitochondrial destruction, altered ventricular expression of fetal gene products, and resulted in selective depletion of cardiac GSH. These TG effects worsened over time, and oxidative stress may be a central subcellular event. The data indicate that Tat depressed cardiac contractility in TGs and could contribute to AIDS CM in patients.

**EXPERIMENTAL PROCEDURES**

**Generation of α-myosin heavy chain/Tat TGs.** Established methods were used. A 1.304-kb HindIII-SmaI fragment containing both exons of Tat (86-residue polypeptide) and an intron from the rat preproinsulin gene (ppI) was isolated from clone pBC12/CMV/Tat-1 (a generous gift from Andrew P. Rice, Univ. of Texas Southwestern Medical Center, Dallas, TX). The fragment was modified with Klenow enzyme (Boehringer, Mannheim, Germany) to fill in the HindIII site. The α-myosin heavy chain (α-MyHC) clone 26 (compliments of Jeff Robins, Children's Research Foundation, Cincinnati, OH; Ref. 39) was digested with SalI and then modified with Klenow enzyme followed by treatment with shrimp alkaline phosphatase (Boehringer) to facilitate construction of the final vector. Restriction analysis and DNA sequencing verified the final construct, denoted α-MyHC/Tat. To generate TGs, a 7.4-kb NsiI-NcoI fragment containing the full transcribing unit was purified and microinjected into FVB one-cell embryos (Charles River, Wilmington, MA). Embryos were incubated in pseudopregnant CD-1 females (Taconic, Germantown, NY). The resulting offspring were screened for incorporation of the transgene. One high-expression hemizygous (+/−Tathigh) and two low-expression hemizygous (+/−Tatlowlow,vA,B) TG lines were created. All mice were housed according to National Institutes of Health guidelines and ad libitum.

**Genotyping.** The α-MyHC/Tat transgene was detected in the founders and their offspring with Southern blotting and PCR. For Southern blotting, 10 μg of mouse genomic tail DNA was digested overnight at 37°C. The digested DNA was subjected to electrophoresis in a 0.7% agarose gel and transferred to a positively charged nylon membrane (Boehringer) to facilitate construction of the final vector. Restriction analysis and DNA sequencing verified the final construct, denoted α-MyHC/Tat. To generate TGs, a 7.4-kb NsiI-NcoI fragment containing the full transcribing unit was purified and microinjected into FVB one-cell embryos (Charles River, Wilmington, MA). Embryos were incubated in pseudopregnant CD-1 females (Taconic, Germantown, NY). The resulting offspring were screened for incorporation of the transgene. One high-expression hemizygous (+/−Tathigh) and two low-expression hemizygous (+/−Tatlowlow,vA,B) TG lines were created. All mice were housed according to National Institutes of Health guidelines and fed ad libitum.

**Protein extraction and Western blot analysis.** Total protein was extracted from 60-day WT, +/−Tathigh, and +/−Tatlowlow,vA hearts with T-PER tissue protein extraction reagent (Pierce, Rockford, IL). Each protein sample (100 μg) was electrophoresed though SDS-18% Tris-HCl polyacrylamide gel (Bio-Rad, Hercules, CA). The resolved proteins were transferred to an Immob-Blot polyvinylidene difluoride membrane (Bio-Rad), blocked with 5% nonfat dry milk in Tris-buffered saline (TBS), and incubated with a mouse monoclonal antibody to HIV Tat (Advanced Biotechnologies, Columbia, MD) 1:4,000 in 2% milk-TBS overnight at 4°C. After four washes in TBS-Tween 20, the membrane was incubated with horseradish peroxidase-linked sheep anti-mouse immunoglobulin G (Amersham, Piscataway, NJ) 1:2,000,000 in 2% milk-TBS for 30 min. The membrane was washed four more times and incubated with SuperSignal West Femto substrate (Pierce). The membrane was exposed to BioMax film (Kodak, Rochester, NY) for 5 min and developed.

**HPLC analysis for antioxidants in heart and quadriceps femoris muscle.** GSH and ascorbate (Asc) were determined with HPLC analysis of the peak corresponding to the signal detected with the wavelengths at 294 and 340 nm. The samples were dissolved in a Tris-EDTA buffer at pH 7.4, and the reaction mixture was incubated at 37°C for 10 min. The reaction was stopped by adding 100 μl of 10% trichloroacetic acid. The supernatants were centrifuged at 10,000g for 10 min, and the supernatants were used for HPLC analysis. The samples were injected onto a C18 reversed-phase column (Zorbax SB C18, 4.6×250 mm, 5 μm) and eluted with a linear gradient of 0.1% trifluoroacetic acid (FA) and acetonitrile (ACN) from 5% FA to 95% ACN over 20 min at a flow rate of 1 ml/min. The eluted samples were detected with a UV detector at 294 nm. The peak areas were quantified using the software provided by the manufacturer. The peaks were assigned as GSH, Asc, and GSSG based on their retention times. The results were expressed as the mean ± SEM.

**References.**

1. K. M. P. Rice, Univ. of Texas Southwestern Medical Center, Dallas, TX.
2. J. Robins, Children's Research Foundation, Cincinnati, OH; Ref. 39.
3. J. A. Dillmann, University of California at San Diego.
4. A. J. Dillman, University of California at San Diego.
5. A. J. Dillman, University of California at San Diego.
6. A. J. Dillman, University of California at San Diego.

**Additional information.**

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mined from a 5-μl injection and based on a five-point standard curve generated with freshly prepared standards.

Echocardiography. Echocardiographic studies were performed serially in WT and +/+ Tathigh and +/+ TatlowA TGs (90, 240, and 365 days) essentially as described previously (21, 30). At least three sequential measurements were obtained (n = 3–16 per cohort).

Transmission electron microscopy. Methods were as described previously (30). Each heart provided ~10 samples for embedding. Myocardium was rinsed in cold Ringer solution and postfixed in 1% OsO4 (Sigma, St. Louis, MO) in PBS, pH 7.4, for 2–3 h. After osmication and rinses, tissue was dehydrated with graded ethanol and embedded in resin (38). Myocardial samples were sectioned (100 nm), stained with uranyl acetate, and examined on a JEOL-JEM-100CX electron microscope. Photomicrographs were enlarged to 8 × 10-in. prints and reviewed for the presence of structurally abnormal mitochondria (as done previously; Ref. 30).

Statistical analysis. Groups were compared by ANOVA as previously described (30). Significance was established with P < 0.05.

RESULTS

Tissue specificity of RNA expression. A transgenic mouse was created that expressed HIV Tat in the heart and that developed a pathophysiological cardiovascular phenotype. Northern analysis of total RNA from different tissues from +/+ Tathigh TGs revealed significant levels of Tat RNA only in the heart and the absence of signal in other tissues (Fig. 1A). Northern analysis of RNA extracted from +/+ TatlowA revealed similar tissue specificity (data not shown).

TAT mRNA expression. Northern analysis of Tat mRNA showed strong signal in blots from +/+ Tathigh and +/+ TatlowA TGs (Fig. 1B). Quantitation of Northern blot signals for cardiac polyadenylated RNA encoding Tat revealed threefold expression of transcribed Tat mRNA in +/+ Tathigh hearts compared with +/+ TatlowA hearts (Fig. 1C). Expression in +/+ Tathigh pup hearts was twice that of +/+ TatlowA TG.

Tat polypeptide expression in WT, +/+ Tathigh, and +/+ TatlowA mice. Extracted polypeptides from hearts of WT, +/+ Tathigh, and +/+ TatlowA mice underwent electrophoresis, transfer, and Western blotting. The data showed Tat polypeptide signal in the Western blots of myocardial extracts from TGs with characteristic electrophoretic mobility of Tat (Fig. 1D). Tat signal was absent in myocardial extract from WT littermates (Fig. 1D). Samples from representative 60-day +/+ Tathigh and +/+ TatlowA mice revealed strong signals in the myocardial extracts (Fig. 1D). Purified Tat served as an external standard (Fig. 1D).

mRNA markers of ventricular remodeling. Characteristic molecular changes of cardiac remodeling were found in RNA extracts from hearts of 180d +/+ Tathigh mice (Fig. 2A). GAPDH was the internal control. By quantitative analysis of the radioactive signals, a twofold increase in steady-state abundance of ANF mRNA was found in ventricular samples from +/+ Tathigh TGs versus WTs (Fig. 2B). Signal for SERCA2 was unchanged. In cardiac ventricular RNA extracts from +/+ Tathigh TGs (60 days), steady-state abundance of ANF was unchanged compared with that of WT littermates (data not shown).

Fig. 1. A: Northern blot analysis of RNA from tissues from high-expression hemizygous (+/+ Tathigh) transgenic mice (TGs). Significant levels of Tat RNA were found in the heart. Conversely, Tat RNA was absent in all other tissues examined. Quad, quadriceps femoris; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B: Northern blot analysis of polyadenylated mRNA in the TG lines. Collage Northern blot follows histogram labels. C: histogram of quantitative molecular data obtained from +/+ Tathigh, +/+ TatlowA, and low-expression hemizygous (+/+ TatlowA) TGs (n = 2 for each). Signal for Tat mRNA in +/+ Tathigh is twofold that of +/+ TatlowA. D: Western blot of extracted myocardial polypeptides from WT, +/+ Tathigh, and +/+ TatlowA purified Tat (external control). No signal for Tat is found in wild-type mice (WT). Extract from +/+ Tathigh and +/+ TatlowA hearts shows clear signal for Tat.
HIV TAT AND CARDIOMYOPATHY

Steady-state abundance of antioxidants in heart and quadriceps femoris muscle. Steady-state abundance of GSH and Asc was determined in heart and quadriceps femoris samples from 120-day +/− Tat\textsubscript{high} TGs and WT littermates at 180 days. GSH abundance was determined in heart and quadriceps femoris muscle. Steady-state abundance of antioxidants in heart and quadriceps femoris muscle samples from +/− Tat\textsubscript{high} mice was unchanged from the respective values in WT littermates (Table 1).

Transmission electron microscopy of heart muscle. Cardiac mitochondrial features were striking and included destruction, enlargement, and loss of cristae in homozygous +/− Tat\textsubscript{high} TGs at 10 days (Fig. 3). In contrast, significant but less prominent mitochondrial structural defects (which worsened with increasing age) were found in hemizygous +/− Tat\textsubscript{high} TGs. At 60 days, samples from +/− Tat\textsubscript{high} TG hearts revealed essentially normal mitochondria. However, at 210–365 days, cardiac mitochondrial damage and enlargement were unambiguous (Fig. 3). Mitochondria from hearts of WT littermates were essentially normal (Fig. 3, bottom). Mitochondria from quadriceps femoris samples were normal in +/− Tat\textsubscript{high} mice (data not shown).

Echocardiographic data from WT, +/− Tat\textsubscript{high}, and +/− Tat\textsubscript{lowA} mice. M-mode echocardiograms were performed and evaluated (single observer) on WT, +/− Tat\textsubscript{high}, and +/− Tat\textsubscript{lowA} mice (90, 240, and 365 days). LV thickening was found in the +/− Tat\textsubscript{high} TGs after as little as 90 days. Quantitatively, +/− Tat\textsubscript{high} TG hearts showed a 46% increase in LV mass at 90 days (P < 0.05), 134% increase at 240 days (P < 0.001), and 96% increase at 365 days (P < 0.001; Fig. 4A). Corresponding changes in LV FS were 28% at 90 days, 27% at 240 days, and 19% at 365 days (P < 0.001 for each comparison to WT littermates; Fig. 4B). Early in life, +/− Tat\textsubscript{high} TG hearts revealed no change in LV mass (30 and 60 days; data not shown). Additionally, at comparable time points up to 365 days, echocardiograms of +/− Tat\textsubscript{lowA} TGs showed no change in LV mass (data not shown).

DISCUSSION

A targeted TG with cardiac-specific expression of HIV Tat was created, and a cardiac pathophysiological phenotype resulted. To our knowledge, this is the first time an HIV gene has been selectively expressed transgenically in cardiac myocytes and the first time a cardiac phenotype resulted from its targeted expression.

In the past, nonspecific TG models of AIDS were generated (23, 24) in which various phenotypes were observed. One well-studied TG, the NL4–3Δ gag/pol (generalized expression of a replication-incompetent HIV construct), exhibited AIDS nephropathy prominently (13) and cardiac dysfunction (30). Recently, a TG rat was generated (with the same NL4–3Δ gag/pol

Table 1. Antioxidants in +/− Tat\textsubscript{high} and wild-type tissues

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<th>TG Heart</th>
<th>WT Heart</th>
<th>TG Q. femoris</th>
<th>WT Q. femoris</th>
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<tr>
<td>GSH</td>
<td>2.9 ± 0.4*</td>
<td>4.7 ± 0.8</td>
<td>15.6 ± 0.8</td>
<td>14.1 ± 0.8</td>
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<td>Asc</td>
<td>0.3 ± 0.2</td>
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<td>2.5 ± 0.1</td>
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Values (in nmol/mg protein) are means ± SE. +/− Tat\textsubscript{high}, high-Tat expression transgenic mice (TG); WT, wild-type mice; Q. femoris, quadriceps femoris; GSH, glutathione; Asc, ascorbate. *P < 0.05.
construct) with AIDS nephropathy prominently and cardiac injury and repair (36). Multisystem disease could impact on cardiac function in these models.

In murine TGs with generalized expression of Tat, phenotypic manifestations included skin lesions, liver disease, lymphoid and other malignancies, and hematologic diseases (7–10, 18, 25, 35, 40). In contrast, our TG lines (operationally defined as \(+/^\text{Tat}_{\text{high}}\)/H11001/H11002/Tathigh, \(+/^\text{Tat}_{\text{high}}\)/H11001/H11001/Tathigh, and \(+/^\text{Tat}_{\text{lowA,B}}\)/H11001/H11002/TatlowA,B) offer the advantage of targeted Tat expression combined with an organ-specific phenotype. This allows us to monitor for organ dysfunction longitudinally in the living animal and to follow its natural history.

The TG lines \(+/^\text{Tat}_{\text{high}}\) and \(+/^\text{Tat}_{\text{lowA}}\) exhibited high and low Tat expression, respectively. \(+/^\text{Tat}_{\text{high}}\) and \(+/^\text{Tat}_{\text{lowA}}\) TG pups developed and matured normally, were fertile, exhibited normal litter size, and survived up to 2 years. In contrast, homozygous \(+/^\text{Tat}_{\text{high}}\) pups died prematurely at \(\sim 14\) days. Ultrastructural pathological examination of hearts from \(+/^\text{Tat}_{\text{high}}\) pups euthanized at 10 days (i.e., \(\sim 4\) days before typical death) revealed no gross structural abnormalities or inflammation. However, profound structural changes in cardiac mitochondria were found by transmission electron microscopy in samples from 10-day \(+/^\text{Tat}_{\text{high}}\) pups.

Pathophysiologic findings in the \(+/^\text{Tat}_{\text{high}}\) TG followed a logical temporal sequence. Data suggest that from 90 to 210 days, cardiac remodeling occurs. At 60
days, robust Tat mRNA was expressed along with Tat polypeptide. At that time, ANF mRNA changes were absent. As early as 90 days, LV FS and LV mass changes of early cardiac dysfunction were found. At 120 days, GSH depletion was evident. At 180 days, ventricular expression of ANF (a sensitive marker of cardiac dysfunction; Ref. 22) was abundant. Transmission electron microscopy mitochondrial changes were unambiguous at 210 days and proceeded to 365 days. Common findings included cristae and matrix disruption, lamellar figures, incomplete fusion, or uninduced mitochondria. These changes paralleled echocardiographic changes of CM. These combined structural and functional changes indicate that Tat caused a mitochondrial CM in which a cumulative threshold effect may be observed (41) that resembles the pathophysiology of some other forms of CM.

The pathophysiology of CM in this model suggests that oxidative stress plays a role. In TGs with ubiquitous Tat expression (driven by the β-actin promoter), inhibition of glutathione synthase (9) and depletion of GSH occurred in the liver. Decreased GSH occurred selectively in +/− Tat<sub>high</sub> TG hearts but not in WT hearts or quadriceps femoris samples from +/− Tat<sub>high</sub> TGs. Asc was unchanged in both heart and quadriceps femoris in any cohort. These findings underscore a relationship between Tat, GSH depletion, and the cardiac-targeted TG phenotype. GSH is an important cellular antioxidant that can directly modulate cellular transcriptional events (1). Additionally, GSH is the only defense available in the mitochondria to metabolize hydrogen peroxide. Thus GSH depletion in this organelle renders cells more susceptible to oxidative stress (16).

M-mode echocardiograms and their quantitative analysis were used to define cardiac dysfunction with age in +/− Tat<sub>high</sub> TGs. At 90 days, +/− Tat<sub>high</sub> TGs exhibited LV enlargement, the earliest indication of cardiac dysfunction. LV enlargement and FS continued for the life of the +/− Tat<sub>high</sub> TG. In contrast, +/− Tat<sub>lowA</sub> LV function was unchanged up to 365 days. Survival in +/− Tat<sub>high</sub> TGs was similar to that of WT littermates for >18 mo.

In summary, we successfully used the α-MyHC promoter to drive HIV Tat gene expression in ventricular cardiac myocytes and created a targeted AIDS TG mouse with cardiac dysfunction and CM. The echocardiographic phenotype began at 90 days and continued throughout life. Features of cardiac dysfunction included cardiomegaly, decreased FS, ventricular expression of ANF (a sensitive marker of cardiac dysfunction), and mitochondrial ultrastructural damage that worsened with age. Homozygote +/+/− Tat<sub>high</sub> pups died at ~14 days with profound cardiac mitochondrial damage. Selective depletion of cardiac GSH links Tat to oxidative stress in this new murine transgenic model of AIDS CM. Future studies with this model may elucidate pathophysiological mechanisms of CM in AIDS and may suggest therapeutic options for treatment or prevention.

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**REFERENCES**


