Myocardial fibrosis and apoptosis, but not inflammation, are present in long-term experimental diabetes

S. Ares-Carrasco,1 B. Picatoste,1 A. Benito-Martín,1 I. Zubiri,1 A. B. Sanz,1 M. D. Sánchez-Niño,1 A. Ortiz,1,2 J. Egido,1,2 J. Tuñón,1,2† and O. Lorenzo1,2†

1Fundación Jiménez Díaz Hospital, and 2Autónoma University, Madrid, Spain

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Ares-Carrasco S, Picatoste B, Benito-Martín A, Zubiri I, Sanz AB, Sánchez-Niño MD, Ortiz A, Egido J, Tuñón J, Lorenzo O. Myocardial fibrosis and apoptosis, but not inflammation, are present in long-term experimental diabetes. Am J Physiol Heart Circ Physiol 297: H2109–H2119, 2009. First published October 9, 2009; doi:10.1152/ajpheart.00157.2009.—The aim of this paper is to study the myocardial damage secondary to long-term streptozotocin-induced type 1 diabetes mellitus (DM1). Normotensive and spontaneously hypertensive rats (SHR) received either streptozotocin injections or vehicle. After 22 or 6 wk, DM1, SHR, DM1/SHR, and control rats were killed, and the left ventricles studied by histology, quantitative PCR, Western blot, ELISA, and electromobility shift assay. Cardiomyocyte cultures were also performed. The expression of profibrotic factors, transforming growth factor-β (TGF-β), connective tissue growth factor, and matrix proteins was increased, and the TGF-β-linked transcription factors phospho-Smad3/4 and activator protein-1 were activated in the DM1 myocardium. Proapoptotic molecules FasL, Fas, Bax, and cleaved caspase-3 were also augmented. Myocardial injury in long-term hypertension shared these features. In addition, hypertension was associated with increased NF-κB, increased inflammatory cell infiltrate, and expression of the mediators [interleukin-1β (IL-1β), tumor necrosis factor-α, monocyte chemoattractant protein 1, vascular cell adhesion molecule 1, angiotensinogen, and oxidants], which were absent in long-term DM1. At this stage, the combination of DM1 and hypertension resulted in nonsignificant additive effects. Moreover, the coexistence of DM1 blunted the inflammatory response to hypertension. Anti-inflammatory IL-10 and antioxidants were induced in long-term DM1 and DM1/SHR hearts. Myocardial inflammation was, however, observed in the short-term model. In cultured cardiomyocytes, IL-10, TGF-β1, and catalase blocked the glucose-stimulated expression of proinflammatory genes. Fibrosis and apoptosis are features of long-term myocardial damage in experimental DM1. Associated hypertension does not induce additional changes. Myocardial inflammation is present in hypertension and short-term DM1, but is not a key feature in long-term DM1. Local reduction of proinflammatory factors and expression of anti-inflammatory and antioxidant molecules may underlie this effect.

hypertension; inflammation; heart

DIABETES MELLITUS (DM) is becoming a pandemic. In 2003, 194 million people had diabetes, and this number is predicted to increase by 72%, affecting 366 million in 2030. Of them, at least 10% have type 1 diabetes (DM1). More than 65% of diabetic patients die because of cardiovascular complications (29, 39). Cardiac damage in DM can be directly induced by DM and enhanced by the coexistence of coronary artery disease and hypertension (2, 14, 21).

Most information on pathogenic mechanisms in diabetic cardiomyopathy is derived from animal models (2, 28). Hypertrophy, fibrosis, apoptosis, and inflammation have been described in the early stages (up to 10 wk) of experimental DM1 myocardial injury (8, 9, 15, 28, 37, 38). The excess of glucose alters metabolic, structural, and contractile proteins and activates cellular responses. Upregulation of the local renin-angiotensin-aldosterone (RAA) system in DM has been shown to be associated with oxidative damage, cardiac cell apoptosis, and interstitial fibrosis (1, 6, 8, 9). Increased reactive oxygen species (ROS) generation and impaired antioxidant defenses could both contribute to oxidative stress in DM hearts (9, 14, 15). Transforming growth factor-β (TGF-β) is another factor overexpressed in DM1 myocardium and involved in fibrotic processes (3, 6, 14, 37). Caspase-3 activation, down-regulation of antiapoptotic molecules, and inflammatory changes, such as leukocyte infiltration and adhesion molecules production, have also been described in the early experimental DM1 myocardium (13, 15, 38). Many of these features are shared by hypertensive cardiomyopathy (9, 19, 27). Some of them, such as the presence of fibrosis, have been confirmed in human myocardial biopsies (21, 35), but there is much less information on features such as local inflammation. The molecular mechanisms involved in these key processes are not fully elucidated, and there is not enough information on the long-term injury of the DM1 heart and its relationship with coexistent hypertension.

In this work, we hypothesized that long-term myocardial injury from persistent hyperglycemia may differ from short-term damage. In this sense, we have studied the fibrotic, apoptotic, and inflammatory events in long- and short-term streptozotocin (STZ)-induced DM1, hypertensive, and DM1/hypertensive myocardium. In long-term DM1, many features of short-term myocardial injury are maintained. However, the inflammatory process appears to be blunted, possibly due to the local expression of anti-inflammatory and antioxidant molecules. Moreover, at this stage, the effects of both combined DM1 and hypertension are, for the most part, not additive.

METHODS

Type 1 diabetic models in rats. Normotensive Wistar-Kyoto and spontaneously hypertensive (SHR) male rats (6 wk of age) received either two STZ injections (50 mg·kg⁻¹·day⁻¹) or vehicle. As a result, there were four different groups (n = 6–10 per group): DM1 normotensive, SHR, DM1/SHR, and control (normotensive) rats. Weight, glycemia, systolic blood pressure (by tail-cuff method), and albuminuria were periodically measured. Final levels are shown in Supplemental Fig. 1. (The online version of this article contains supplemental data.) Insulin (1–4 IU sc, Insulatard NPH) was administrated...
weekly to prevent death, but keep blood glucose >400 mg/dl. After 22 wk of treatment (long-term DM1), rats were killed, and the left ventricles isolated. One-half of the sample was included in 4% paraformaldehyde for histological studies, and the other one-half was frozen in liquid N₂ for protein and RNA assays. Serum creatinine, measured at death, remained within normal limits in all groups (0.5 ± 0.08 mg/dl). Some 6-wk-old rats were similarly treated with STZ and followed for 6 wk (short-term DM1), and their left ventricles analyzed. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996) and was approved by the Ethical Committee of the Hospital.

Cardiomyocyte culture. H9c2(2-1) is a permanent cardiomyocytes cell line derived from embryonic BD1X rat heart tissue (ATCC). Cells were grown at 37°C in 5% CO₂ in Dulbecco’s modified Eagle’s medium (BioWhittaker, Verviers, Belgium) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (BioWhittaker), 100 IE/ml sodium penicillin (Yamanouchi Europe, Leiderdorp, The Netherlands), 100 μg/ml streptomycin (Radiopharma-Fistopharma), 2 mM l-glutamine (GIBCO-BRL, Paisley, UK), and 5 mM d-glucose (Sigma). Properties of H9c2 cells are similar to those in adult cardiomyocytes (5). An interesting feature of this cell line is its ability to differentiate from mononucleated myoblasts to myocyte on reduction of serum concentration. Accompanying myocyte formation is the expression of myogenic transcription factors and calcium channel proteins. During this differentiation process, cells retain several elements of the electrical and hormonal signaling pathway of cardiac cells and have, therefore, become an accepted in vitro model to study the effects of diabetes on the heart (36). To prevent loss of myoblastic properties, cultures were subcultured before they become confluent, and the line recloned periodically with selection for myoblastic cells. In the experiments, 80–85% confluence cells (0.9 × 10⁶ cells/cm²) were used in serum-depleted media. Hyperglycemia was mimicked by adding d-glucose up to the final concentration of 33 mM. This glucose concentration corresponds to plasma levels of 590 mg/dl. For coinubation studies, recombinant human TGF-β₁, rat interleukin (IL)-10 (20 ng/ml, Peprotech) and/or catalase (50–500 U/ml, Sigma) were added together with the high-glucose medium. Total RNA was studied by quantitative PCR (QPCR).

Histology and immunohistochemistry techniques. Myocardium samples were embedded in paraffin. Four-micrometer paraffin sections were stained with Masson trichrome, Sirius red, and hematoxylin-eosin (H/E) following the manufacturer’s instruction (Bio-optical). Cardiac fibrosis was quantified on Sirius red-stained sections with Image pro Plus software. Stained area-to-total area ratio (×100) of an average of 10 fields is indicated in Fig. 1. Cell size was quantified as cell diameter of the average of 50 randomly H/E-stained cells. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was used to detect apoptosis (CardioTACSTM, R&D Systems). Apoptotic cells presented blue nuclear staining. Negative control was made without dUTP-transferase enzyme (not shown). A semiquantitative estimation of apoptotic cells defined as TUNEL-positive cells was carried out by Metamorph software. Data show the percentage of stained-positive nuclei as average of 10 fields in the myocardium.

For immunohistochemistry (IH), endogenous peroxidase activity was blocked with 3% methanol-H₂O₂. Unspecific sites were treated with a blocking buffer (5% albumin and 10% specific serum in PBS). Primary antibody [anti-collagen I (Calbiochem), anti-CTGF (conective tissue growth factor; Torrey Pines), anti-Cd68 or anti-Cd3 (for macrophages and T lymphocytes, respectively; Serotec)] was added to the sections in blocking buffer and incubated overnight at 4°C. After washing, secondary biotin-labeled antibody was added, washed, and developed with AB streptavidin-complex and diamobenzidine chromogen. Infiltrate was quantified with Metamorph software on 10 fields of myocardium. Graph shows the number of CD68 and CD3 positive leukocytes per area (mm²) of myocardium. In all cases, photographs were taken at ×40 magnification. A scale bar is shown in each photograph.

Protein assays (Western blot, ELISA, and electromobility shift assay). Briefly, a piece of frozen half ventricle was cold pulverized, dissolved in lysis buffer (50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 2% SDS + 1/250 protease inhibitors), and vortexed for 30 min on ice. After centrifugation at 13,000 rpm, supernatant (total protein extract) was separated and quantified by the bicinchoninic acid method (Pierce). Equal amounts of samples (40 μg) were loaded on an acrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked with 5% fat-free milk in Tris-buffered saline + Tween 20 0.05% and incubated with the primary antibody [anti-TGF-β (Abcam), -Fas, -FasL (Fas ligand; Sta. Cruz bt.), -Bax (Pharmingen), -Bcl2 (Sta. Cruz bt.), -cleaved-Asp175-caspase3 (Cell Signalling), -catalase (Calbiochem), -IL-6 (Abcam), -IL-10 (R&D systems), or -TNF-α (Abcam)] overnight at 4°C. Next, membranes were incubated with horseradish peroxidase-labeled secondary antibody and developed with ECL/X-ray films (Amersham). α-Tubulin or GAPDH (Sigma) was the loading control. In Figs. 1–5, we show a representative gel of three experiments of all rats and the semiquantification score (n-fold). The protein molecular weights are indicated. Enzyme-linked immunosorbent assay (ELISA; Bender Medystems) was performed for TGF-β₁, (ref.: BMS623) and IL-10 (ref.: BMS629) in cell culture supernatants. H9c2 were incubated with 33 mM d-glucose for 24–48 h, and media was collected and analyzed following manufacturer’s instructions. To block protease activity, media was supplemented with 1% fetal bovine serum.

Electrophoretic mobility shift assay was used to detect nuclear transcription factor activation. Briefly, a frozen piece of ventricle was pulverized, dissolved in lysis buffer (20 mM HEPES, pH 7.5, 20% glycerol, 0.35 M NaCl, 5 mM MgCl₂, 0.1 mM EDTA, 1/250 cocktail protease inhibitors), and vortexed for 30 min on ice. Protein quantification was made as explained above. commercial oligonucleotides [phospho-Smad3/4 (p-Smad3/4) and activator protein-1 (AP-1) from Promega] were labeled with [γ-³²P]ATP, incubated 30 min with cellular extracts in binding buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol) and separated on acrylamide gels. Gels were run, dried out, and exposed to X-ray films. Protein (nuclear transcription factor) and oligonucleotide binding was semi-quantified by densitometry. Competition assay for specific binding was made preincubating the protein extract with the unlabeled or unrelated (not shown) oligonucleotide, before adding the labeled oligonucleotide. In Figs. 1–5, we show a representative gel of three experiments of all rats and the semiquantification score (n-fold). The protein-oligonucleotide complexes are indicated. All complexes for each transcription factor were semiquantified together.

QPCR. Analysis of the relative gene expression was performed by retro-transcription and QPCR. Total RNA from left myocardium or cultured cells was dissolved in TRizol reagent (Invitrogen). After RNA extraction, quality and quantity were measured by absorbance (260- to 280-nm absorbance ratio). Equal amounts of total RNA were reverse transcribed to obtain the cDNA, which was used for multiplex QPCR. The reaction was prepared as follows: for each point, 33 ng of cDNA, 0.25 μl of gene expression assays (0.125 μl target gene + 0.125 μl housekeeping gene), 5 μl premix buffer (polymerase and salts), and RNase free water (up to 10 μl). Applied Biosystems expression assays (Taqman Fam fluorophore) were as follows: angiotensinogen (Ao) (Rn00593114_m1), CTGF (Rn00573960_m1), fibronectin (FN) (Rn00569575_m1), IL-1β (Rn00580432_m1), heme oxygenase 1 (HO-1) (Rn01536933_m1), monocyte chemotaxtractant protein 1 (MCP-1) (Rn00580555_m1), nitric oxide synthase (NOS)-1 (Rn00587393), and VCAM-1 (Rn00563627_m1). Housekeeping fuekaryotic ribosomal 18S was Vic fluorophore (4310893E). Amplification conditions were as follows: 2 min at 50°C, 10 s at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C (Applied B. 7500). All samples were prepared in triplicate to obtain their threshold cycle. If deviation for each triplicate were higher than 0.3 cycles, threshold
cycle was not considered. In each figure, we show a quantification (fold gene vs. 18s) of at least three QPCRs of all rats or cultured cardiomyocytes.

**Statistics.** Results are expressed as means ± SD. For in vitro experiments, this represented at least three independent experiments. Multiple comparisons were performed by a Kruskal-Wallis test followed by a Mann-Whitney test. A two-tailed *P* < 0.05 was considered significant.

**RESULTS**

**Characterization of the long-term DM1 and hypertensive rat model.** STZ is a pancreatic β-cell toxin used to induce chronic and severe hyperglycemia, consistent with experimental DM1 in adult rats (30). In our model, rats were killed 22 wk after DM1 induction. Normotensive STZ-induced DM1 animals presented hyperglycemia (554 ± 28 mg/dl blood glucose) and albuminuria (1,578 ± 314 μg/day) (Supplemental Fig. 1A). DM1/SHR rats also showed hyperglycemia (450 ± 65 mg/dl), severe albuminuria (7,804 ± 2,810 μg/day), and high-systolic blood pressure (201 ± 3 mmHg). SHR rats showed higher blood pressure (204 ± 1.8 mmHg) but similar albuminuria than DM1 rats (1,570 ± 147 μg/day). Control rats kept normal blood glucose level (80 ± 2.8 mg/dl), blood pressure (138 ± 11.6 mmHg), and albuminuria (176 ± 4.3 μg/day). As expected, DM1 rats gained significantly less weight compared...
with normotensive and SHR rats (Supplemental Fig. 1A). In the short-term (6 wk) model, STZ-treated animals presented also hyperglycemia and albuminuria (Supplemental Fig. 1B).

**Myocardial fibrosis induced by long-term STZ-induced DM1 and hypertension.** Masson trichrome, Sirius red, and H/E staining were used to examine the morphological changes in the myocardial interventricular septum. The myocardium from control animals showed normal structure (Fig. 1A). However, both DM1 and DM1/SHR rats presented an increased extracellular matrix deposition, as stained by Sirius red (7.5 and 11.2% for DM1 and DM1/SHR, respectively) (Fig. 1, A and B). Fibrosis was mainly interstitial and perivascular. IH indicated that type I collagen was a main component of the fibrotic extracellular matrix (Fig. 1A). In addition, in DM1 and DM1/SHR myocardium, FN mRNA expression was elevated (1.8- and 2.3-fold, respectively, vs. control) (Fig. 1C). Interestingly, SHR rats showed similar fibrosis and matrix protein expression as DM1/SHR (Fig. 1). As previously observed (6, 8), extracellular matrix deposition was also detected in the myocardium of short-term (6-wk) STZ-induced DM1 (1.76%) and hypertensive (5.97% for SHR and 2.03% for DM1/SHR) rats vs. control (1.2%) (Fig. 2; Sirius red).

On the other hand, myocardial cell size was increased in long-term DM1 (124.3 ± 1.25% vs. control) and more conspicuously in both SHR and DM1/SHR rats (170.3 ± 1.7% and 208.7 ± 1.87%, respectively, Fig. 1A; H/E). A lesser increase in cell size was noted in short-term DM1, SHR, and DM1/SHR (113.77 ± 3.47, 144.0 ± 3.01, and 137.77 ± 2.5%, respectively, Fig. 2; H/E). However, the hypertrophic effect on these pathologies was not an objective of this study.

**Long-term DM1 and hypertension promote a proapoptotic milieu in the myocardium.** By TUNEL staining, long-standing STZ-induced DM1, SHR, and DM1/SHR rats presented a significant increase of apoptotic cells in the myocardium (Fig. 3A). DM1 hearts showed 5.08 ± 1.2% apoptotic nuclei, whereas SHR and DM1/SHR depicted 11.1 ± 2.5 and 14.0 ± 1.5%, respectively ($P < 0.01$ vs. control). As expected, lower apoptotic levels were found in short-term DM1 and hypertensive animals (0.79, 1.63, and 1.22%, respectively. Fig. 2; TUNEL).

The apoptotic mechanisms have not been elucidated in long-term DM1. TNF superfamily proteins, such as FasL, promote apoptosis through caspase activation (7, 22). The expression of FasL and its receptor (Fas) was increased in the DM1 (1.43- and 2.3-fold, respectively, vs. control) and DM1/SHR (2.05- and 1.69-fold, respectively, vs. control) myocardium (Fig. 3B). Moreover, the Bax (proapoptotic)-to-Bcl2 (antiapoptotic) ratio was higher in the DM1 (7-fold vs. control, Fig. 3C, bottom) and DM1/SHR myocardium (5.7-fold vs. control). Finally, activation of caspase-3 in DM1 and DM1/SHR was also augmented (3- and 3.5-fold, respectively, vs. control) (Fig. 3D). The SHR rats presented

![Fig. 2. Short-term STZ-induced DM1 and hypertensive injury in the heart. Fibrosis (by Sirius red), hypertrophy (H/E), apoptosis [terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)], and inflammation (CD68+ detection) representative photographs are shown. Arrowheads indicate apoptotic nuclei or inflammatory cells (macrophages) in each panel.](https://www.ajpheart.org/molecular-mechanisms-in-the-long-term-diabetic-myocardium)
Fig. 3. DM1, hypertension, and cardiac apoptosis. A: TUNEL assay stained apoptotic cells (arrow heads) in long-term STZ-induced DM1, SHR, and DM1/SHR myocardium. Semiquantification of apoptosis is showed as percentage of apoptotic nuclei (blue) vs. total nuclei (×100). By Western blot, Fas ligand (FasL) and Fas (B); pro- and antiapoptotic Bax, Bcl2, and Bax/Bcl2 ratio (C); and caspase-3 (cleaved-activated protein) (D) are shown. Semiquantitative scores (n-folds) for each protein are also indicated on the right. *P < 0.01 or **P < 0.05 vs. control. ***P < 0.01 vs. DM1.
similar levels of apoptotic-related factors than DM1/SHR (Fig. 3).

The TGF-β system in long-term DM1 and SHR myocardium. The TGF-β system is a key mediator of fibrosis in the heart and other tissues through deposition of extracellular matrix proteins, such as FN and type I collagen, from local cells (13a, 26, 34). In addition, TGF-β may increase the expression of lethal molecules, such as Fas (23). In long-term STZ-induced DM1 and SHR myocardium, TGF-β₁ protein levels were augmented (3.2 and 3.3-fold, respectively, vs. control, Fig. 4A). When DM1 was associated with hypertension, there was a further increase in TGF-β₁ levels (4.8-fold, \( P < 0.01 \) vs. control, \( P < 0.05 \) vs. SHR and \( P = \) nonsignificant vs. DM1).

Fig. 4. Long-term DM1, hypertension, and myocardial transforming growth factor (TGF)-β system. A: myocardial TGF-β₁ protein levels by Western blot. Semiquantification (n-folds) for each protein is also shown (right). B: connective tissue growth factor (CTGF) myocardial staining and mRNA expression. A representative QPCR amplification (delta Rn vs. cycle) of a rat of each group (control, DM1, SHR, DM1/SHR) for 18s and CTGF is also shown. C: by EMSA, TGF-β₁-linked phosphor-Smad3/4 (p-Smad3/4), and activator protein (AP)-1 activated complexes (arrows) in STZ-induced DM1, SHR, and DM1/SHR myocardium. Semiquantification for each transcription factor is shown (right). C, means specificity competition assay (unlabeled oligonucleotide). Nonspecific probe was similar for all rats (not shown). *\( P < 0.01 \) or **\( P < 0.05 \) vs. control, **\( P < 0.05 \) vs. SHR, and ***\( P < 0.01 \) vs. DM1.
CTGF is a well-known mediator of the TGF-β system actions (17, 32), although its expression has not been described in DM. CTGF expression was increased (mainly in myocytes) in long-standing DM1, SHR, and in DM1/SHR myocardium (IH and PCR; 3.6-, 4.2-, and 3.5-fold, respectively, vs. control mRNA expression, Fig. 4B).

We also studied the activation of TGF-β-linked transcription factors p-Smads and AP-1. Using specific oligonucleotides for both Smad3/4 (against p-Smad3 and -Smad4 members) and AP-1, we detected activated transcription complexes (Fig. 4C, arrows). Four p-Smads complexes and one AP-1 complex were observed in the myocardium. Competition assay with cold oligonucleotides demonstrated the specificity of the binding (Fig. 4C, C). In long-term STZ-induced DM1 and SHR myocardium, all four p-Smads complexes were increased (2.9- and 5.2-fold, respectively, \( P < 0.01 \) vs. control). Added hypertensive enhancement in long-standing DM1 effect (7.5-fold, \( P < 0.01 \) vs. DM1 and control). AP-1 was also activated in the three groups (4.2-, 4.6-, and 5.4-fold, respectively, \( P < 0.01 \) vs. control).

**Inflammation in long-term DM1 and SHR myocardium.** The presence of myocardial inflammation has been previously described in SHR (19, 27) and short-term STZ-induced DM1 (38). We now confirmed these data in short-term DM1 rats by CD68 staining (for macrophage detection). At this stage, DM1, SHR, and DM1/SHR myocardium showed inflammatory cell recruitment (3.77, 3.58, and 1.88 cells/mm², respectively) (arrowheads, Fig. 2; CD68⁺). However, in the long-standing model, we did not observe significant CD68 or CD3 positive cells (for T lymphocytes) infiltrating cells, both in DM1 and DM1/SHR myocardium (Fig. 5A). Increased expression of proinflammatory cytokines IL-1β and TNF-α was described in short-term myocardial injury (38). In our long-term DM1 model, IL-1β, TNF-α, MCP-1, IL-6, and VCAM-1 were not significantly increased in DM1 hearts (Fig. 5B). However, we confirmed increased expression of TNF-α in short-term DM1 heart (1.92-fold vs. control, Fig. 5C). TNF-α was also induced in both long- and short-term SHR (2.05- and 1.61-fold, respectively, vs. control; Fig. 5C) and short-term DM1/SHR myocardium (1.65-fold vs. control, Fig. 5C).

In addition, proinflammatory factor NF-κB, which regulates the expression of many of these genes, was not activated in long-term DM1 (Fig. 5D). In addition, DM1 showed a dominant effect on NF-κB activation, since its presence attenuated the hypertensive effects (Fig. 5D).

**Anti-inflammatory cytokines and antioxidants in experimental DM1 heart and glucose-incubated cardiomyocytes.** We examined the expression of anti-inflammatory and antioxidant molecules in long-term DM1 myocardium. IL-10 was stimulated in long-term DM1 (3.73-fold vs. control), but not in short-term DM1 (Fig. 5, B and C). The anti-oxidants catalase (Fig. 5, B and C) and HO-1 (Fig. 5E) presented a similar induction only in long-term DM1 (2.31- and 3.59-fold, respectively, vs. control). Accordingly, the prooxidant neuronal NOS-1 was not elevated in the DM1 heart (Fig. 5E). Long-term SHR hearts presented increased IL-10 (2.97-fold vs. control) and HO-1 (6.13-fold vs. control), unchanged levels of catalase, and elevated NOS-1 (1.67-fold vs. control) (Fig. 5, B and E). In short-term SHR myocardium, IL-10, catalase, and HO-1 were decreased or unchanged (Fig. 5, C and E), whereas NOS-1 was stimulated (Fig. 5E). All of these effects were attenuated by the coexistence of both pathologies in DM1/SHR rats (Fig. 5, B, C, and E).

In addition, we evaluated the presence of a local activated RAA system. The expression of the angiotensin II precursor, Ao, was reduced in both long- and short-term DM1 (Fig. 5E). In contrast, Ao was enhanced in short-term SHR rats (1.62-fold vs. control), and this response disappeared again in DM1/SHR hearts.

In vivo findings suggest that long-term DM1 may activate secondary mediators, which dampen the myocardial inflammatory mechanism, and diminished proinflammatory responses to hyperglycemia. We next studied whether cultured cardiomyocytes could secrete anti-inflammatory and antioxidant molecules when exposed to long incubation of high-glucose medium. By ELISA, we detected both TGF-β and IL-10 molecules in H9c2 media after 48 h of high-glucose incubation (Fig. 6A). The increase in IL-10 secretion lagged behind that of TGF-β. Thus we analyzed the direct effect of exogenous TGF-β and IL-10 on high-glucose-induced proinflammatory responses. High glucose induced also proinflammatory MCP-1 (5 min to 1 h) and VCAM-1 (5 min to 6 h) expression in cardiomyocytes (Fig. 6B). The maximal MCP-1 expression induced by high glucose was significantly decreased by coinoculation with anti-inflammatory TGF-β or IL-10 (0.33- and 0.35-fold, respectively vs. high glucose alone at 30 min). Both TGF-β and IL-10 also reduced VCAM-1 expression induced by high glucose (0.22- and 0.49-fold, respectively, vs. 3-h high glucose alone). Incubation with both TGF-β and IL-10 in high-glucose medium abolished MCP-1 peak expression. VCAM-1 mRNA was similarly reduced (Fig. 6B). Finally, catalase was also added to the high-glucose media to the cardiomyocytes. After 15–30 min of incubation, catalase (150–300 U/ml) normalized MCP-1 and VCAM-1 glucose-induced expression in a similar manner than the anti-inflammatory cytokines (0.43- and 0.4-fold, respectively, vs. high-glucose alone).

**DISCUSSION**

Fibrosis and apoptosis are important events in the DM heart. Both responses have been described in human (13, 21, 35) and short-term experimental DM1 (our data, Refs. 2, 8, 9, 37, 38) and hypertension (19, 27). We now confirm that fibrosis and apoptosis are also features of long-term STZ-induced DM1 and hypertension. TGF-β1 can be a primary mediator for both fibrosis and apoptosis (13a, 23, 34). Via AP-1 activation, TGF-β1 is involved in cardiac hypertrophic growth, apoptosis, and fibrosis (34). Through Smads proteins, TGF-β1 also increases extracellular matrix deposition by induction of ROS and calcium influx (34). TGF-β1 activates caspases and the Fas pathway (23), and TGF-β1-induced CTGF leads to cancer cell apoptosis through a diminution of Bcl2 expression (17). In both short- and long-term DM1, TGF-β1 overexpression has been described (3, 37). We have observed increased TGF-β expression in long-standing experimental DM1 in association with an increased profibrotic (collagen I, FN, CTGF) and proapoptotic (Bax and Fas) factors and enhanced activity of TGF-β-linked transcription factors (AP-1 and p-SMADs). Thus activation of the TGF-β pathway may underlie the extracellular matrix deposition and apoptosis in the long-term DM1 and hypertensive myocardium. In addition, hypertrophy was also stimulated in these hearts and could be linked to the observed cell apoptosis (and matrix deposition). It is noteworthy that
TGF-β is also an anti-inflammatory cytokine (22) and may be involved in the control of cardiac inflammation.

In human studies, nonspecific serum inflammatory markers, such as IL-6, TNF-α, troponin, or C-reactive protein, suggesting systemic inflammation, have been linked to cardiovascular dysfunction in diabetic patients (25, 30, 33). However, there is a lack of information showing local inflammatory leukocytes on human myocardial biopsies or necropsies. In this sense, our results demonstrated that long-standing STZ-induced DM1 and SHR cardiomyopathies differed in a key feature. Long-term DM1 myocardium did not present inflammatory cells, while the inflammatory infiltrate, expression of inflammatory mediators (TNF-α, IL-1β, MCP-1, and VCAM-1) and activation of the proinflammatory transcription factor NF-κB were prominent in long-term SHR heart injury. However, increased TNF-α expression and macrophage infiltration were noted in short-term DM1 heart injury. These results are in agreement with a recent work, which shows unchanged IL-6, VCAM-1, and MCP-1 expression in the rat myocardium from 7 to 24 wk after type 2 diabetes (11).

In cardiomyocytes, we also show that a high-glucose environment induces the release of TGF-β, which can modulate proinflammatory gene expression. Nevertheless, it is unlikely that TGF-β is the only factor suppressing inflammation in the long-term STZ-induced DM1 heart, since its expression and actions (such as p-Smad and CTGF activation) are increased also in hypertensive cardiomyopathy. In the heart, both cardiomyocyte and noncardiomyocyte cells can express anti-inflammatory IL-10 (40), which is increased in spleen and heart, but not in liver, thymus, or bone of diabetic rats (24). Opposite to short-term DM1, we have detected an increased expression of IL-10 in long-standing DM1 myocardium (parallel to an unchanged TNF-α and IL-6 expression). In long-term SHR hearts, although IL-10 was also elevated, there was overexpression of proinflammatory TNF-α. In addition, IL-10 was also released from cultured cardiomyocytes after high-glucose stimulation. In this sense, in DM1 patients, blood mononuclear cells released more IL-10 than controls, and this was associated with a decrease of proinflammatory IL-1, IL-6, and TNF-α secretion (12). In our cell cultured experiments, IL-10 secretion...
was delayed in a temporal manner with respect to TGF-β. Indeed, exogenous IL-10 decreased the inflammatory response induced by high-glucose media, and this effect was further enhanced by TGF-β. These data are also consistent with the observation that, in cardiomyocytes, IL-10 antagonizes IL-1β and IL-6 expression, ROS production, and inflammation promoted by TNF-α (4, 20). Thus IL-10 may contribute to the absence of inflammation observed in long-term DM1 heart. In this sense, IL-10 has been classified as a protective interleukin in the cardiovascular system (10). Decreased IL-10 plasma levels are associated with a high incidence of cardiovascular events (16). Moreover, systemic induction of IL-10 expression in Dahl salt-sensitive rats reduced inflammatory infiltration, hypertrophy, and cardiac dysfunction in the hypertensive heart (27).

The oxidative state may play a role in the cardiac inflammation related to DM1. Activation of RAA system during DM1 is associated with increased oxidative damage and cardiomyocyte death, which contributes to the increased interstitial fibrosis and inflammation (2, 6, 9). Blockade of the RAA system in STZ-treated rats attenuates cardiac dysfunction, partially through the reduction of ROS production (6, 25, 37). Importantly, the change in the expression of RAA system components in DM1 hearts appears to be local and independent of the circulating RAA system. Thus STZ-induced DM1 heart apoptosis peaked at 3 days and decreased after 28 days, in correlation with a reduction in RAA system components (8, 14). In this sense, and in contrast to hypertension, we have observed a decrease in Ao and unchanged NOS-1 expression in long-term DM1 hearts. In parallel, antioxidants catalase and HO-1 were overexpressed. In cardiomyocytes, catalase also decreased proinflammatory glucose-induced genes. Thus, together with the increase of the anti-inflammatory-to-proinflammatory ratio, long-term DM1 heart may develop compensating mechanisms to dampen myocardial damage. Nevertheless, more experimental models targeting anti-inflammatory and antioxidant molecules are needed to fully confirm their potential therapeutic role in DM cardiac disease. In this regard, Ao expression is not always directly associated to increase RAA system activity and NOS-1 may inhibit xanthine oxidoreductase, which is responsible for ROS production (2a).

Finally, the concurrence of both DM1 and hypertension pathologies worsens the deleterious effect of either disease alone and accelerates cardiovascular mortality in humans (13, 21). However, at short- and long-term experimental stages, there was not a consistent additive effect when both disorders are experimentally combined. SHR and DM1/SHR rats showed similar fibrosis, apoptosis, and expression of related proteins. More interestingly, in our work, the presence of DM1 dampened the inflammatory changes observed in SHR animals. A concordant previous report showed that 1-wk STZ treatment does not further increase myocardial contractile dysfunction, oxidative state, and apoptosis in SHR rats (9).

**Study limitations.** An echocardiographic examination would have been useful to provide a functional correlation of the histological and molecular abnormalities found. In this sense, it has been demonstrated previously in rats that both systolic and diastolic dysfunctions are evident after 6 wk of DM1 (38). Our long-term rats were analyzed after 22 wk of DM1.

**Conclusions.** We demonstrated that long-standing STZ-induced DM1 is characterized by fibrosis and apoptosis in the heart, in association with the activation of the TGF-β system. These effects were independent of hypertension and not enhanced by its presence. Long-standing DM1 cardiomyopathy differs also from early DM1 and long-term hypertensive myocardial injuries by the absence of an inflammatory response. Anti-inflammatory molecules expressed in the DM1 heart (and by high-glucose exposed cardiomyocytes), such as IL-10 and TGF-β, together with reduced local TNF-α, RAA/nitric oxide systems modulation, and antioxidant production, may account for this finding. Further investigations should address the therapeutic consequences of these differences in the treatment of DM1 and hypertensive heart damage.

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**DISCLOSURES**

No conflicts of interest are declared by the author(s).

**REFERENCES**


