Targeted disruption of ICAM-1, P-selectin genes improves cardiac function and survival in TNF-α transgenic mice


Targeted disruption of ICAM-1, P-selectin genes improves cardiac function and survival in TNF-α transgenic mice. Am J Physiol Heart Circ Physiol 280: H1464–H1471, 2001.—We have developed a transgenic mouse model in which tumor necrosis factor (TNF-α) is overexpressed exclusively in the heart under the regulation of the α-myosin heavy chain promoter. These animals develop chronic heart failure associated with severe leukocyte infiltration in both the atria and the ventricles. The purpose of this study was to investigate the role of adhesion molecules in mediating cardiac dysfunction in the TNF-α transgenic model. TNF-α transgenic mice were bred with mice null for intercellular adhesion molecule (ICAM)-1 and P-selectin genes to obtain a lineage of ICAM-1 and P-selectin null mice with selective overexpression of TNF-α in the heart. TNF-α transgenic animals showed marked upregulation of ICAM-1 mRNA and protein; however, P-selectin mRNA and protein remained undetectable despite chronic TNF overexpression. Cardiac function was markedly improved in the ICAM-1−/−, P-selectin−/−, TNF-α transgenic group versus the ICAM1+/+, P-selectin+/+, TNF-α transgenic group. Kaplan-Meier survival curves showed statistically significant prolonged survival in the ICAM-1−/−, P-selectin−/−, TNF-α transgenic animals. These data suggest that ICAM-1 mediates at least in part the cardiac dysfunction induced by TNF-α expression by cardiac myocytes.

α-myosin heavy chain promoter (TNFαg+/–) (3). These animals develop severe chronic heart failure. Histological examination of the hearts showed neutrophilic, monocyctic, and lymphocytic infiltration consistent with transmural myocarditis. One possibility is that this inflammatory infiltrate is secondary to necrotic myocytes and thus is unrelated to the pathogenesis of cardiac dysfunction. However, it is also possible that myocyte TNF-α production facilitates the adhesion and transmigration of leukocytes and that these leukocytes mediate cardiac injury through release of oxygen free radicals and proteases. The recruitment of leukocytes from the circulation by the endothelium is essential for the initiation and targeting of an inflammatory response (25). Selectins mediate the initial rolling of leukocytes along the endothelium of the vessel wall (1, 2, 21, 26, 30, 37). Leukocyte rolling is closely followed by an increase in the avidity of the adhesion molecules of the integrin family, leading to firm adhesion of the cells to the endothelium. Firm adhesion of leukocytes is mediated by binding of the β2-integrin family to the immunoglobulin superfamily of adhesion molecules [intercellular adhesion molecule (ICAM)-1, ICAM-2, and vascular cell adhesion molecule-1], which are expressed in the vascular endothelium (6, 9, 12, 22, 35). Previously, several investigators (31, 39) demonstrated that both P-selectin and ICAM-1 are highly induced by TNF-α, and inhibition of either or both of these molecules limits leukocyte transmigration into various organs. The purpose of our study was to investigate the role of P-selectin and ICAM-1 in mediating leukocyte transmigration as well as the impairment of cardiac function in our TNF-α transgenic model.

METHODS

Animals

All animals were used in accordance with the guidelines of the University of Texas Southwestern Medical Center Animal Care and Research Advisory Committee and in compliance with

Address for reprint requests and other correspondence: B. P. Giroir, Children’s Medical Center of Dallas, 1935 Motor St., Dallas, TX 75325 (E-mail: bgiroiro@childmed.dallas.tx.us).

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the rules governing animal use as published by the National Institutes of Health. TNF-α transgenic mice were obtained as previously described (3). ICAM-1 knockout (TNF-α) mice (C57BL/6-ICAM<sup>fl/fl</sup> Sel<sup>fl/fl</sup>) were purchased from Jackson Laboratories (Bar Harbor, ME). ICAM-1 transgenic (double knockout, TNF<sup>fl/fl</sup>-<sup>α</sup>) mice were bred with TNF-α transgenic to obtain a colony of ICAM-1−/−, P-selectin−/− mice with overexpression of TNF-α in the heart. Four groups of animals were studied: 1) C57BL/6 wild-type (WT) mice; 2) ICAM-1−/−, P-selectin−/− (double knockout) mice; 3) TNF-α transgenic (TNF<sup>fl/fl</sup>-<sup>α</sup>) mice; and 4) ICAM-1−/−, P-selectin−/−, TNF<sup>fl/fl</sup>-<sup>α</sup> transgenic (double knockout, TNF<sup>fl/fl</sup>-<sup>α</sup>) mice. Briefly, ICAM-1−/−, P-selectin−/− female mice were bred to TNF<sup>fl/fl</sup>-<sup>α</sup> males. The resulting heterozygous ICAM-1−/−, P-selectin−/− mice, which was confirmed by PCR (ICAM-1 and TNF) and Southern blots (P-selectin). The colony was maintained by sibling mating. Weights were obtained weekly. Animals were anesthetized and euthanized by cervical dislocation at 21, 40, and 75 days of life. These time points were selected because animals were weaned at 21 days and severe cardiac dysfunction is observed by 75 days; 40 days was chosen as an intermediate time point in which most of the animals appeared clinically healthy.

**Survival**

WT and double knockout (control groups), double knockout TNF<sup>fl/fl</sup>-<sup>α</sup> (n = 18 mice/group), and TNF<sup>fl/fl</sup>-<sup>α</sup> (n = 48 mice) were followed for 150 days for survival. This time interval was set at twice the historical survival rates of TNF<sup>fl/fl</sup>-<sup>α</sup> mice. Animals that appeared hunched, tachypneic, or in distress were euthanized. Data were plotted into Kaplan-Meier survival curves.

**Screening**

Transgenic offspring were identified by PCR amplification of unique transgene sequences from tail DNA as previously described (3). P-selectin screening was done by Southern blot analysis (4). The probe for P-selectin was kindly provided by Dr. Albert Beaudet of Baylor College of Medicine, Houston, TX. Genotyping of the mouse ICAM-1 gene was accomplished by a PCR protocol from Jackson Laboratories. Briefly, a common primer (OP2: 5′-GAGGGCGAGGCGAAAAAGAACG-3′) was paired with either the selectable marker (OP1: 5′-AGGAGACAGGACGGGAGGAGATT-3′) or a primer from within exon 5 (12292: 5′-CTGAGGGCGTGGGATCGTCCG-3′) to amplify either a 150-bp product from the mutant allele or a 178-bp product from the WT allele.

**Probe for Northern Blots**

Probes were generated in our laboratory by the following method: RNA was isolated from the mouse lung harvested after 2 h of lipopolysaccharide stimulation. RNA (1 μg) was reverse-transcribed with SuperScript II RT (GIBCO-BRL; Grand Island, NY) into cDNA. The following primers were then used to PCR the appropriate fragment; each of these fragments was then cloned into plasmide pCR2.1 (Invitrogen) and amplified. The ICAM probe was exon 5: 5′-GTCTTCTGAGGCGCGCT-3′; exon 7: 5′-AGAAGCCTCAGTCTCG-3′ (34). The P-selectin probe was exon 6: 5′-ACAGGTGGCAGCAGTGTC-3′; exon 2: 5′-CGGAGCTTCGAGCTGGAAGTT-3′ (4).

**Northern Blot Analysis**

Hearts (n = 5 hearts/study group) were isolated, rinsed in cold PBS, immediately frozen in liquid nitrogen, and stored at −80°C. RNA isolation was performed using the TRIzol method (GIBCO-BRL). RNA was quantified by ultraviolet spectrophotometry at 260/280 nm. Total RNA (5 μg) was mixed with 2× RNA loading buffer and denatured at 65°C for 10 min. Electrophoresis was performed in a standard 1% agarose gel and transferred to a nylon hybridization membrane (Hybond-N+, Amersham Pharmacia; Piscataway, NJ). The RNA was cross-linked to the membrane with short-wave ultraviolet light (GS Gene Linker, Bio-Rad Laboratories; Hercules, CA). The membrane was placed in a solution containing 50% formamide, 5× Denhardt’s solution, 0.1% SDS, 5× sodium chloride-sodium phosphate-EDTA, and 100 μg/ml denatured fragmented salmon sperm DNA. Prehybridization was carried out in a shaking water bath for 1 h at 55°C for ICAM-1 and 60°C for P-selectin. The appropriate probes were random labeled with [α-<sup>32</sup>P]dCTP (Ready To Go, Amersham Pharmacia Biotech), boiled for 2 min, chilled for 1 min, and then added to the hybridization solution. Hybridization was carried out overnight at 55°C for ICAM-1 and 60°C for P-selectin. The blots were washed at 5°C higher than hybridization temperature as follows: 2× saline sodium citrate (SSC) + 0.1% SDS for 30 min, 1× SSC + 0.1% SDS for 30 min, and 0.2× SSC + 0.1% SDS for 30 min. Autoradiography was performed with intensifying screens at −80°C for 24 h.

**Antibodies for Western Blots**

The rabbit anti-human P-selectin polyclonal antibody (CD62P) was purchased from PharMingen (San Diego, CA). Goat anti-human ICAM-1 polyclonal antibody (M-19) as well as rabbit and goat horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Western Blots**

For Western blots, n = 3 hearts/group. Total protein content was determined using a Bio-Rad Protein Assay. Fifty micrograms of protein per sample were added to an equal volume of 2× sample buffer (100 mM Tris-HCl, 2% SDS, 0.02% bromophenol blue, and 10% glycerol) and boiled for 5 min. Electrophoresis was performed in a standard 10% SDS-PAGE gel. Proteins were then electrophotoically transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Temecula, CA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline and incubated with the appropriate antibodies. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. The enhanced chemiluminescence signals were visualized with ChemiDoc XRS (Bio-Rad). The blots shown are representative of three independent experiments.

**Fig. 1.** Representative Northern blot for intercellular adhesion molecule (ICAM)-1 showing upregulation in the heart of tumor necrosis factor (TNF)-α transgenic (TNF<sup>fl/fl</sup>-<sup>α</sup>) animals at 21, 40, and 75 days. ICAM-1 was not detected in wild-type (WT) animals, double knockout (KO) ICAM−/−, P-selectin−/− animals, or double KO TNF<sup>fl/fl</sup>-<sup>α</sup> animals. Heart tissue harvested after 2 h of intraperitoneal injection of lipopolysaccharide (LPS) was used as a positive control.
**Fig. 2.** Representative Western blot showing increase synthesis of ICAM-1 protein at 21, 40, and 75 days. No protein synthesis was observed in WT animals, double KO ICAM−/−, P-selectin−/− animals, or double KO TNF−/− animals. Heart tissue harvested after 2 h of intraperitoneal injection of LPS was used as a positive control.

**Fig. 3.** Representative Northern (top) and Western blot (bottom) for P-selectin. P-selectin expression was detected only in the LPS (2 h)-stimulated heart (Northern) and spleen (Western) used as positive controls.

**Cytokine Profile by RT-PCR**

RNA isolation. Total RNA was isolated from hearts taken from 40-day-old animals (~50 mg wet wt) using TRIzol (GIBCO-BRL) according to the manufacturer’s instructions. Tissues were analyzed in blinded fashion. RNA isolation.

Langendorff Preparations of Isolated Perfused Hearts

Mice were anticoagulated with 100 units of heparin sodium and cervically dislocated. The heart was rapidly removed and placed in ice-cold Krebs-Henseleit bicarbonate-buffed solution [containing (in mM) 118 NaCl, 4.7 KCl, 21 NaHCO₃, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, and 11 glucose]. All solutions were prepared on the day of performance with demineralized deionized water and bubbled with 95% O₂-5% CO₂ (pH 7.4, PO₂ 583 mmHg, and PCO₂ 38 mmHg). The ascending aorta was cannulated, and the catheter was subsequently connected to a Krebs-Henseleit bicarbonate reservoir for perfusion. A flow pump (model 911, Holter) was used to maintain a constant flow rate of 1.5 ml/min. Hearts were suspended in a temperature-controlled chamber (38 ± 0.5°C). The bicarbonate...
perfusate was passed through a heating coil maintained at 38 ± 0.5°C before delivery to the aorta. A pressure transducer connected to the tubing between the heart and the heating coil was used to measure coronary perfusion pressure. Effluent was collected and measured to confirm coronary flow rate. Intraventricular pressure was measured with a saline-filled polyethylene tube threaded into the left ventricular (LV) chamber. LV pressure (LVP) was measured with a Statham P231D pressure transducer attached to the cannula. LV change in pressure over time (dP/dt) values were obtained using an electronic differentiator (model 7P20C, Grass Instruments). All parameters were recorded on an ink writing recording system (model 7DWL8P, Grass Recording Instruments). Starling relationships were determined by plotting LV systolic pressure and maximum rise and fall in dP/dt (+dP/dt max and −dP/dt max, respectively) values against incremental increases in either coronary flow rate or Ca²⁺ concentrations in the perfusate. Because heart rate varied, hearts were paced as required by an electrode attached to the right atrium (4.8–5.0 A for 1-ms duration; Grass Stimulator) (15).

Statistics
Analysis was performed using SPSS for Windows (version 7.5.1).

Cardiac function. All values are expressed as means ± SE. ANOVA was used to assess an overall difference among the groups for each of the variables. Levene's test for equality of variance was used to suggest the multiple comparison procedure to be used if the ANOVA was significant. If equality of variance among the four groups was suggested, multiple comparison procedures were performed (Newman-Keuls or Bonferroni). If inequality of variance was suggested, Thamane's multiple comparisons were performed. P values < 0.05 were considered statistically significant.

Survival. A Kaplan-Meier survival analysis was performed on the survival data.

RESULTS

ICAM-1 and P-selectin mRNA and Protein Expression in Hearts of TNFtg+/− Mice

We investigated ICAM-1 and P-selectin expression in the hearts of 21-, 40-, and 75-day-old TNFtg+/− and WT animals. ICAM-1 mRNA and protein expression were markedly upregulated in the TNFtg+/−
mice at all time points (Figs. 1 and 2) compared with WT aged-matched controls; ICAM-1 was not detected in double knockout or double knockout TNFtg+/- animals. P-selectin expression was not detected by Northern or Western blots in any of the study groups (Fig. 3).

**Cytokine Profile by RT-PCR**

To determine whether the expression of cytokines was altered by the targeted disruption of the ICAM-1 and P-selectin genes, we performed semiquantitative RT-PCR with the heart tissue of 40-day-old animals. The amplification of RNA encoding β-actin confirmed that equivalent amounts of RNA were analyzed in each reaction. TNF-α, IL-1β, and IL-6 were equally upregulated in TNFtg+/- and double knockout TNFtg+/- mice (Table 1).

**Cardiac Function**

Cardiac function was evaluated by Langendorff preparations of hearts obtained from 70-day-old animals (n = 10 mice/group). There was no difference in the time interval from death to initiating perfusion between control and experimental groups. Cardiac performance was not altered in double knockout animals (ICAM-1−/−, P-selectin−/− mice). No difference in cardiac function was observed between both control groups (double knockout and WT mice) (Fig. 4). Thus, to simplify graphs, data from control groups were combined compared with TNFtg+/- animals. Significant cardiac dysfunction was observed in TNF-α transgenic animals (TNFtg+/-), which seems to be partially mediated by ICAM-1 and P-selectin. The targeted disruption of ICAM-1, P-selectin genes (double knockout TNFtg+/- mice) significantly improved cardiac function. (LVP 97.7 ± 6.6 vs. 72.6 ± 5.0 mmHg; +dP/dt max 2,214 ± 171 vs. 1,716 ± 118 mmHg/s; and −dP/dt max 1,793 ± 104 vs. 1,420 ± 143 mmHg/s) (P < 0.05). This improvement in cardiac function in double knockout TNFtg+/- animals is shown by a shift of the curve upward and to the left (Figs. 5 and 6). Stepwise increases in coronary flow rate improved contractile performance in all hearts at each time point regardless of the experimental group assignment. However, function was always decreased in the TNFtg+/- group compared with the double knockout TNFtg+/- group. Similarly, stepwise increases in the per-

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**Coronary Flow Rate (ml/min)**

Fig. 5. Control flow rate in control, double KO TNFtg+/-, and TNFtg+/- mice. Isolated perfused heart (Langendorff) preparations showing severe cardiac dysfunction in TNFtg+/- mice and statistically significant improvement in cardiac function in double KO TNFtg+/- animals when compared with TNFtg+/- animals (see text for reference values). *P < 0.05, control vs. TNFtg+/- animals. +P < 0.05, double KO TNFtg+/- vs. TNFtg+/- animals. +dP/dt max and −dP/dt max, maximum rise and fall in the change of pressure over time.
Fusate Ca$^{2+}$ concentration improved contractile function in all hearts at each time point; however, function was always decreased in the TNF$^{tg}$ group compared with the double knockout TNF$^{tg}$ mice.

Survival

All animals were phenotypically normal at birth and remained so until the onset of a characteristic illness in double knockout TNF$^{tg}$ animals. Control groups (WT and double knockouts) remained normal throughout the experimental period. TNF$^{tg}$ and double knockout TNF$^{tg}$ animals eventually developed a clinical syndrome of decreased activity, tachypnea, and edema or weight loss. However, double knockout TNF$^{tg}$ animals had a statistically significant longer survival period when compared with TNF$^{tg}$ animals: the result of the log rank test indicated significant group differences in survival between TNF$^{tg}$ and double knockout TNF$^{tg}$ mice, with the estimated median number of survival days for TNF$^{tg}$ mice at 80 days [95% confidence interval (CI): 74–86] significantly lower than that for the double knockout TNF$^{tg}$ mice at 120 days (95% CI: 112–128) ($P < 0.001$). Control animals (WT and double knockouts) demonstrated 100% survival throughout the study period of 150 days (Fig. 7).

DISCUSSION

In this study, we demonstrated that TNF-α upregulates ICAM-1 but not P-selectin in the myocardium of...
TNF-α transgenic animals and that the targeted disruption of the ICAM-1, P-selectin genes delays heart failure and improves survival in this animal model.

The notion that TNF-α is involved in cardiac disease evolved from observations concerning the pathogenesis of cardiac dysfunction during septic shock (29). Several lines of evidence also suggested a role of TNF-α in other cardiac-related diseases. Circulating levels of TNF-α are increased in patients with chronic heart failure (17, 20). Matsoumori et al. (24) reported elevated serum levels of TNF-α in patients with acute myocarditis, dilated cardiomyopathy, and hypertrophic cardiomyopathy. In addition, increased TNF-α expression in intramyocardial blood vessels and cardiac myocytes has been shown in heart biopsies of patients with dilated cardiomyopathy (10). Increased TNF-α levels have also been reported in heart transplant rejection and after cardiopulmonary bypass.

The mechanism by which TNF-α causes heart failure has not yet been elucidated. Our previous results (3) clearly indicate that myocardial production of TNF-α is sufficient to cause myocarditis and severe heart failure. However, those experiments did not distinguish whether damage is directly caused by TNF-α, by inflammatory cells that have been recruited by TNF-α, by the expression of other cytokines, or by the induction of nitric oxide synthases and the generation of free radicals, or by other mechanisms not yet understood.

The expression of ICAM-1 has been demonstrated in cardiac myocytes in both humans with unexplained cardiac dysfunction and animals with acute and healing myocarditis as well as in myocardial tissue of children with lymphocytic myocarditis (16, 33). In addition, inhibition of ICAM-1, P-selectin, or both has been effective in minimizing cardiac dysfunction after ischemia-reperfusion injury, viral infection, heart transplant, and cutaneous thermal injury (11, 14, 32, 36, 40).

Previous studies have suggested that there is redundancy of function between ICAM-1 and P-selectin, such that in knock-out animals chronic compensatory expression of one gene may mitigate the effects of disrupting the other gene. In such circumstances, the pathophysiological importance of cell migration might be masked. Because our primary purpose was to determine whether cell adhesion mediates, at least in part, the fatal heart failure in this model, utilization of a double knockout lineage (ICAM-1 and P-selectin) was indicated. However, results indicated that only ICAM-1 mRNA and protein are indeed upregulated, whereas neither P-selectin mRNA nor protein was detected at any time point.

In addition, we demonstrated that targeted disruption of the ICAM-1 and P-selectin genes attenuated the degree of cardiac dysfunction and improved survival in this animal model. Because P-selectin was not detectable in controls at any time point, it is reasonable to conclude that improved survival is primarily, if not exclusively, related to the disruption of the ICAM-1 gene.

Although ICAM-1 expression is involved in the pathogenesis of heart failure in this model, its involvement is incomplete in that mortality was improved but not prevented. Incomplete protection may reflect the underlying pathophysiology but may also reflect the limitations of knockout models. Mice deficient in adhesion molecules (either ICAM-1, P-selectin, or both) from the time of embryogenesis may utilize alternative adhesion pathways that can compensate for the missing molecules. The molecules that mediate these alternative pathways are not yet clear. In addition, recently novel isoforms of murine ICAM-1 have been described in ICAM-1 mutant mice (18, 38). These isoforms have been shown to bind the ICAM-1 counter-receptor leukocyte function-associated antigen (LFA-1). At least part of the cardiac dysfunction in our model could be explained by one of these ICAM-1 isoforms.

The mechanism of TNF-α-induced heart failure is probably multifactorial. The expression of other adhesion molecules, chemoattractant receptors, and chemokine receptors as well as specific mediators that are released may determine the type of cells that will infiltrate the myocardium and the degree of inflammation and myocardial damage. There is little known about the expression of adhesion molecules under chronic TNF exposure. Acute versus chronic exposure to TNF may have different effects in the expression of inflammatory mediators. Previous studies (31, 39) reported induction of P-selectin mRNA and protein under acute TNF stimulation. To our knowledge, our study is the first one to examine the expression of ICAM-1 and P-selectin under chronic TNF stimulation of the heart. Our results show that the lack of ICAM-1 activity attenuates cardiac dysfunction and prolongs survival in this animal model. On the basis of these results, we believe that ICAM-1 may be a target for therapeutic interventions in myocarditis and other TNF-α-related cardiac diseases. Further studies are planned to define the molecular mechanism of myocardial dysfunction in TNF-α transgenic animals, including the role of ICAM-1 and P-selectin independently, the role of other adhesion molecules (e.g., vascular cellular adhesion molecule-1), and the role of other cytokines like IL-1β and IL-6, which were shown to be increased in the animals that overexpressed the TNF transgene.

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