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Cytokine Polymorphisms and Histologic Expression in Autopsy Studies: Contribution of TNF-α and TGF-β1 to the Pathogenesis of Autoimmune-Associated Congenital Heart Block

Robert M. Clancy,* Chelsea B. Backer,* Xiaoming Yin,* Raj P. Kapur,† Yair Molad,‡ and Jill P. Buyon2,8

Although Abs to SSA/Ro-SSB/La are necessary for the development of congenital heart block (CHB), the low frequency suggests that fetal factors are contributory. Because CHB involves a cascade from inflammation to scarring, polymorphisms of the TNF-α promoter region and codons 10 and 25 of the TGF-β gene were evaluated in 88 children (40 CHB, 17 rash, 31 unaffected siblings) and 74 mothers from the Research Registry for Neonatal Lupus (NL). Cytokine expression was assessed in autopsy material from two fetuses with CHB. Significantly increased frequency of the −308A (high-producer) allele of TNF-α was observed in all NL groups compared with controls. In contrast, the TGF-β polymorphism Leu10 (associated with increased fibrosis) was significantly higher in CHB children (genotypic frequency 60%, allelic frequency 78%) than unaffected offspring (genotypic frequency 29%, allelic frequency 56%, p = 0.016; allelic frequency 56%, p = 0.011) and controls, while there were no significant differences between controls and other NL groups. For the TGF-β polymorphism, Arg25, there were no significant differences between NL groups and controls. In fetal CHB hearts, protein expression of TGF-β, but not TNF-α, was demonstrated in septal regions, extracellularly in the fibrous matrix, and intracellularly in macrophage infiltrates. Age-matched fetal hearts from voluntary terminations expressed neither cytokine. TNF-α may be one of several factors that amplify susceptibility; however, the genetic studies, backed by the histological data, more convincingly link TGF-β to the pathogenesis of CHB. This profibrosing cytokine and its secretion/activation circuitry may provide a novel direction for evaluating fetal factors in the development of a robust animal model of CHB as well as therapeutic strategies in humans. The Journal of Immunology, 2003, 171: 3253–3261.
can bind cognate Abs; macrophages phagocytose these opsonized apoptotic cardiocytes and secrete inflammatory cytokines such as TNF-α (10). Other investigators have also demonstrated that phagocytosis of opsonized apoptotic cells is proinflammatory (12, 13). Primary culture of human fetal cardiac fibroblasts exposed to supernatants obtained from macrophages incubated with opsonized apoptotic cardiocytes markedly increased the expression of the profibrogenic myofibroblast marker smooth muscle actin (SMαc) (14). The addition of TGF-β-neutralizing Abs to the opsonized supernatant blocked expression of SMαc (14). Support for cross talk between macrophages and fibroblasts is further supported by histologic evidence of a SMαc-positive infiltrate and macrophages in areas of scar tissue in the heart of a term male infant diagnosed with AV block at 19 wk of gestation and dying at birth (14).

Taken together, these data support a role for cytokines in generating and sustaining the inflammation and fibrosis that amplify the pathologic effects of maternal autoantibodies in favor of cardiac scarring. TNF-α is a proinflammatory cytokine. Recently, a biallelic polymorphism was found at position −308 within the human TNF-α promoter. The guanine (G) to arginine (A) substitution at position −308 has been associated with increased production of the cytokine (15, 16). The common (wild-type) allele, −308G (TNF1), has a frequency of ∼80% in Caucasians and 92% in African-Americans (17). TGF-β1 is a multifunctional cytokine that modulates the proliferation and differentiation of many cell types, and is considered to play a critical role in fibrotic conditions (18). The polymorphisms at codon 10 (leucine→proline) and codon 25 (arginine→proline) are associated with interindividual variation in the levels of TGF-β1 production. For both polymorphisms, the alleles encoding proline are generally associated with lower TGF-β1 synthesis in vitro and in vivo (19). The frequencies of the Leu10 and Arg25 alleles in Caucasians are 64 and 90%, respectively (19, 20). Accordingly, the present study was initiated to address whether frequencies of select polymorphisms in the TNF-α and/or TGF-β1 genes are increased or decreased in children with CHB compared with unaffected anti-SSA/Ro-exposed fetuses (siblings) and healthy controls. A complementary approach involved examination of cardiac sections from fetuses dying with CHB, and healthy fetuses electively terminated, for mRNA and protein expression of these cytokines.

Materials and Methods

Genetic studies

Study subjects from NL families. The family members included in this study were enrolled in the Research Registry for Neonatal Lupus, established in September 1994 by the National Institute for Arthritis and Musculoskeletal and Skin Diseases and extensively described (21). Consent (approved by the Institutional Review Board of the Hospital for Joint Diseases/New York University Medical Center) to participate in the Registry and forward serum samples, DNA, and medical records was obtained from all mothers and their children if older than 18 years. For inclusion in the present study, the fetus, neonate, or child was considered to have CHB if the following two criteria were met: 1) heart block (first, second, or third degree) documented by electrocardiogram, echocardiogram, history of pacemaker, or doctor’s note stating CHB; and 2) presence of Abs to the 52-kDa SSA/Ro, 60-kDa SSA/Ro, or 48-kDa SSB/La ribonucleoproteins in the maternal serum, as determined by ELISA (Diamedix, Miami, FL), ELISA with recombinant proteins, and SDS immunoblot to identify the fine specificity of the autoantibody response. A child was considered to have cutaneous manifestations of NL if the following two criteria were met: 1) photograph demonstrating the characteristic annular lesions and/or clear description of the rash in the medical records and/or skin biopsy characteristic of NL (basal cell vascuolopathy and a mononuclear cell infiltrate); and 2) maternal autoantibodies documented equivalent to the criteria for CHB above. A child was considered to be a healthy sibling if: 1) there were no cardiac, cutaneous, hepatic, or hematologic manifestations of NL, and maternal autoantibodies were documented equivalent to the criteria for CHB above. A mother was included in the cohort if she had: 1) at least one child with any manifestation of NL, and 2) autoantibodies, as described above. The ethnic breakdown of the groups was: mothers (n = 74), 82% Caucasian, 4% Hispanic, 8% African-American, 6% Asian; children with CHB (n = 40), 82% Caucasian, 8% Hispanic, 8% African-American, 3% Asian; children with rash (n = 17), 100% Caucasian; and unaffected children (n = 31), 84% Caucasian, 6% Hispanic, 3% African-American, 6% Asian.

Control populations. Controls used for TNF-α included an historical cohort described by Vinasco et al. (22), comprising 102 healthy volunteer blood donors of a Caucasian population in northern Europe with a male:female ratio of 1:1 (84, −308G/G; 16, A/G; 2, A/A), and a second historical cohort described by Louis et al. (23) comprising 98 healthy Caucasian controls (69, −308G/G; 27, A/G; 2, A/A). Controls used for TGF-β1 included an historical cohort described by Awad et al. (19) comprising 107 healthy volunteer blood donors of a Caucasian population of laboratory workers and renal transplant donors (Codon 25, 87 Arg/Arg, 19 Arg/Pro, 4 Pro/Pro). Codon 10, 44 Leu/Leu, 51 Leu/Pro, 12 Pro/Pro). A second control cohort for TGF-β1, described by Holweg et al. (20), comprised 94 healthy volunteers (>95% Caucasian, male:female ratio 1:1) (Codon 25, 79 Arg/Arg, 15 Arg/Pro, 0 Pro/Pro; Codon 10, 37 Leu/Leu, 43 Leu/Pro, 14 Pro/Pro).

DNA analysis. DNA was isolated from anticoagulated blood using the Qiagen kit (QiAamp DNA Blood Mini Kit, Valencia, CA), following the instructions of the manufacturers. PCR-RFLP was selected to genotype the TNF-α −308 region. In brief, PCR consist of 50–100 ng of DNA in 1 μl, 2 μl of dNTP at 10 mM, 10 μl 10× Taq buffer, 3 μl of 50 mM MgCl2, 5 μl of formamide, 10 μl 5 mM 5′-AGGCAATAGGTTTTGGGTTAGG-3′ (NcoI-TNF-α promoter F-169), 10 μl 3 μl 5′-ACATCCCCACATCCTGTCGTCTCC-3′ (TNF-α promoter R-285), and 1 μl AmpliTaq polymerase in a final volume of 100 μl. Cycling conditions were as follows: 94°C for 5 min; 40 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; 72°C for 10 min. Complete, 8 μl of product was digested with 1 μl restriction enzyme buffer and 1 μl of NcoI overnight at 37°C. Products were separated electrophoretically on a 10% acrylamide Tris-borate-EDTA gel. The polymorphism associated with high levels of TNF-α production (−308A/A) resists NcoI digestion and yields a 117-bp band. The polymorphism associated with lower levels of TNF-α production (−308G/G) cuts to produce a 97-bp fragment.

An allele-specific PCR was used to genotype the exon 1 region of TGF-β1, which contains both codon 10 and codon 25. The PCR conditions for amplification of genomic DNA were identical with that described for TNF-α, except that primers selective for TGF-β1 were substituted: the sense primer sequence is 5′-TTCAGACCAACCACTCTTCT-3′ (TGF-β1-F-1734); the antisense primer sequence is TCGCGGGTGCTGTTGTACA (TGF-β1-R-2233). To assess codons 25 and 10, the amplified DNA (5–10 μl) was then digested in a 20 μl reaction mixture, with the enzymes FseI (2.0) and MspAI (10 U), respectively. Digestions were conducted for at least 2 h, and products were fractionated in 7% polyacrylamide gels. For codon 25, the 500-bp ampiclon does not cut with FseI, if there is a cytosine. For the guanine-containing polymorphisms, two fragments are produced: 318 and 182 bp. To assure that the restriction enzymes were active and that a cytosine was not incorrectly assigned, DNA from established homozygotes G/G and heterozygotes G/G and heterozygotes was included in each set of reactions. For codon 10 of TGF-β1, DNA was digested with MspAI, which results in a 285-bp fragment if thymidine is present, and a 273-bp fragment if cytosine is substituted.

Statistical analysis. Fisher’s exact test was used to determine whether differences in the prevalence of the allele and genotypic frequencies between different groups of subjects were statistically significant at p values <0.05 (2 × 2 contingency table) (see Tables I and II).

Autopsy sections

CHB cases. The clinical description and gross anatomy of the two CHB cases have been previously described (24). Briefly, one was a female fetus diagnosed with third degree block at 22 wk gestation and electively terminated. The mother was asymptomatic and received no treatment. The second case was a female fetus diagnosed at 18 wk with third degree block and hydrops; fetal demise occurred 20 wk gestation despite several days of maternal oral dexamethasone at 4 mg/day. The mother had previously been diagnosed with Sjogren’s syndrome. The serum from both mothers of these affected fetuses contained anti-SSA/SSB/La Abs. Neither heart was grossly malformed.
Controls. Hearts were obtained following elective termination of three fetuses (22, 23, and 24 wk gestation) in which there was no known cardiac disease, and from a term newborn dying of noncardiac causes.

Preparation of slides. Formalin-fixed paraffin sections (6-μm section) were obtained from all hearts. Sections of right ventricle, left ventricle, and AV groove/conduction (septal) tissue were obtained from the 20-wk CHB, 22-wk CHB, and 23-wk control hearts. Right and left ventricular sections were obtained from the 22- and 24-wk control hearts.

Oligonucleotide in situ hybridization. In situ hybridization was performed by Zephan Biopharmaceuticals, Baltimore, MD) (25). Deparaffinized sections of heart tissue were treated with RNase-free proteinase K (25° C, followed by a 1-h prehybridization period (prehybridization mix: 0.2 mg/ml tRNA; 1 mmol/L sodium pyrophosphate; 5 mmol/L EDTA; 10% dextran sulfate). Sections were hybridized at 42°C overnight with antisense cDNA (Maxim Biotech, South San Francisco, CA). Brieﬂy, probe (2 μg/ml) was commercially prepared by PCR fragments of the pGem-T vector carrying a full-length human TGF-β1 insert (using T7 RNA-polymerase (Roche)). After extensive washing of the tissue sections, hybridization was visualized using anti-digoxigenin, alkaline phosphatase-conjugated Abs (Roche), and Fast Red TR/Naphthol AS-JMX Tablet (Sigma-Aldrich; D-4168) or Fast Red TR/Naphthol AS-JMX Tablet sets (Sigma-Aldrich; F-4648) to report peroxidase and alkaline phosphatase activity, respectively. The sections were counterstained with hematoxylin before examination and photomicroscopy.

Staining for fibrosis. The picrosirius red staining method was chosen because this approach accurately reflects organ collagen content assessed with hydroxyproline assays (26).

Results

The −308 promoter polymorphism of TNF-α in children with CHB, children with rash, unaffected offspring, and their mothers Evaluation of the cohort revealed a significantly increased frequency of the −308A allele in all members of the NL families relative to published controls (Tables I and II).

Table I. Frequency of TNF-α polymorphisms in families with neonatal lupus vs controls

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Allelic frequency</th>
<th>Genotypic frequency</th>
<th>p vs controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−308A (%)</td>
<td>A/A (%)</td>
<td>A/G (%)</td>
</tr>
<tr>
<td>CHB children (n = 40)</td>
<td>26</td>
<td>5</td>
<td>43</td>
</tr>
<tr>
<td>NL rash children (n = 17)</td>
<td>32</td>
<td>0</td>
<td>65</td>
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<tr>
<td>Unaffected children (n = 31)</td>
<td>24</td>
<td>0</td>
<td>35</td>
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<tr>
<td>Mothers (n = 74)</td>
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<td>4</td>
<td>66</td>
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<tr>
<td>Controls (Ref. 22) (n = 102)</td>
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<td>2</td>
<td>16</td>
</tr>
<tr>
<td>Controls (Ref. 23) (n = 98)</td>
<td>16</td>
<td>2</td>
<td>28</td>
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Table II. Frequency of TGF-β polymorphisms in families with neonatal lupus vs controls

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<th>p vs controls</th>
</tr>
</thead>
<tbody>
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<td>Leu10 (%)</td>
<td>Leu/Leu (%)</td>
<td>Leu/Pro (%)</td>
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<tr>
<td>CHB (n = 40)</td>
<td>78a</td>
<td>60b</td>
<td>35</td>
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<td>64</td>
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<td>47</td>
</tr>
<tr>
<td>Unaffected (n = 31)</td>
<td>56a</td>
<td>29b</td>
<td>55</td>
</tr>
<tr>
<td>Mothers (n = 74)</td>
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<td>43</td>
<td>43</td>
</tr>
<tr>
<td>Controls (Ref. 19) (n = 107)</td>
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<td>41</td>
<td>48</td>
</tr>
<tr>
<td>Controls (Ref. 20) (n = 94)</td>
<td>62</td>
<td>39</td>
<td>46</td>
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<table>
<thead>
<tr>
<th>Subjects</th>
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<th>Genotypic frequency</th>
<th>p vs controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arg25 (%)</td>
<td>Arg/Arg (%)</td>
<td>Arg/Pro (%)</td>
</tr>
<tr>
<td>CHB (n = 40)</td>
<td>94</td>
<td>88</td>
<td>12</td>
</tr>
<tr>
<td>NL rash (n = 17)</td>
<td>88</td>
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<tr>
<td>Unaffected (n = 31)</td>
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<td>Controls (Ref. 19) (n = 107)</td>
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<td>18</td>
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<tr>
<td>Controls (Ref. 20) (n = 94)</td>
<td>92</td>
<td>84</td>
<td>16</td>
</tr>
</tbody>
</table>

* TGF-β polymorphism Leu10 (associated with increased organ fibrosis) was distributed significantly differently in the children with CHB (genotypic frequency 60%, allelic frequency 78%) compared with the unaffected offspring (genotypic frequency 29%, p = 0.016; allelic frequency 56%, p = 0.011).
frequency of the −308A allele in the mothers was 37% (55 of 148) ($p = 0.0001$ vs controls), in children with CHB 26% (21 of 80) ($p = 0.003$ vs controls), and in children with rash 32% (11 of 34) ($p = 0.004$ vs controls). These results appear to be a reflection of simple inheritance because the unaffected offspring had a similar frequency (15 of 62, 24%, $p = 0.029$ vs control). For all family members, there was a significant decrease in the −308G/G genotypic frequency compared with controls. Although the children with rash had the lowest −308G allelic and genotypic frequency of the children, the results did not reach statistical significance when compared with the children with CHB or unaffected offspring. Analysis of the data eliminating the non-Caucasians did not change the results.

The Leu$^{10}$ and Arg$^{25}$ polymorphisms of TGF-β in children with CHB, children with rash, unaffected offspring, and their mothers

In contrast to the results for TNF-α, polymorphism at codon 10 of TGF-β was distributed significantly differently in the children with CHB compared with unaffected offspring. Specifically, in CHB the Leu/Leu genotypic frequency was 24 of 40 (60%) and Leu allelic frequency 62 of 80 (78%), compared with the unaffected offspring in whom the genotypic frequency was 9 of 31 (29%) ($p = 0.016$) and allelic frequency 35 of 62 (56%) ($p = 0.011$). There was also a significant increase in the genotypic and allelic frequency of Leu$^{10}$ in the children with CHB compared with the population controls (81 of 201, 40%, $p = 0.0241$; 256 of 402, 64%, $p = 0.02$). These frequencies remained significant when the non-Caucasian children with CHB were removed from the analysis. There were no significant differences in either genotypic or allelic frequencies between children with rash, unaffected children, or mothers and the controls.

For codon 25 of TGF-β, the Arg$^{25}$ allele was widely expressed with no significant differences in either the genotypic or allelic frequency in any of the NL family groups compared with controls.

**FIGURE 1.** TNF-α mRNA expression in conduction tissue of fetus with CHB and control. A and C, Show longitudinal sections through the septum of a 20-wk fetus with CHB; B and D, show longitudinal sections through the septum of a normal 23-wk abortus. Oligonucleotide in situ hybridization was performed using the sense and antisense TNF-α digoxigenin-labeled probes. mRNA is reported using peroxidase-conjugated anti-digoxigenin.

**FIGURE 2.** Histological evidence of increased fibrosis in conduction tissue of two fetuses with CHB and colocalization with TGF-β and myofibroblasts in CHB. A, C, and D, Longitudinal sections through the septum of a 20-wk fetus with CHB, 22-wk fetus with CHB, and a 23-wk control, respectively. Picrosirius stain was used to identify collagen (red). Enhanced fibrosis is detected in cardiac sections obtained from the fetuses with CHB, but not healthy control. B, A section adjacent to that represented in A was double stained with anti-human TGF-β (peroxidase) and anti-SMAc (alkaline phosphatase) to demonstrate the proximity of TGF-β, myofibroblasts, and fibrosis.

Evaluation of cytokines in histologic sections of two fetuses with CHB

TNF-α was not observed by immunostaining in either of the two fetal hearts with CHB or the heart of an electively terminated normal control of similar gestational age. However, expression of TNF-α mRNA was demonstrated in both CHB hearts and localized to a region near the AV groove at a site enriched in mononuclear cells (Fig. 1). There was no detectable expression of TNF-α in the control heart or in CHB hearts with treatment, which omitted the antisense cDNA (data not shown).

To evaluate the extent of fibrosis, cardiac sections were stained with picrosirius red for the detection of collagen. In both CHB hearts, there was extensive fibrosis in the inferior portion of the atrial wall, where the AV node is likely to reside (Fig. 2). Collagen deposition was absent in the septal tissue of the healthy control. In both the 20- and 22-wk CHB fetuses, TGF-β immunoreactivity was seen in the conduction tissue (Figs. 3 and 4). In several sections, intense TGF-β staining was present in the extracellular fibrous matrix between the concentrated SMAc-positive myofibroblasts in the adjacent subendocardium and infiltrating CD68-positive macrophages (Figs. 3 and 4). In this location, staining appeared to be extracellular. In both the 20-wk (Fig. 4) and the 22-wk heart (data not shown), double labeling revealed colocalization of TGF-β in the cytoplasm of macrophages, including multinucleate giant cells. TGF-β was also localized in cells in areas of intact myocardium adjacent to conduction tissue (data not shown). The...
latter scattered cells were not recognized by specific Abs to markers of macrophages or myofibroblasts (data not shown). Expression of TGF-β mRNA was more focused than the diffuse pattern of TGF-β immunostain (Fig. 5). Specifically, transcripts were detected in two areas near the AV groove in regions containing mononuclear cells (H&E staining; data not shown). No staining was seen in the 20-wk CHB hearts with treatment, which omitted the antisense cDNA (data not shown). There was no fibrosis, TGF-β immunostain, or mRNA present in conduction tissue or ventricles from the control 22- and 23-wk abortuses.

Because Smad2 is a transcription factor that translocates from the cytoplasm to the nucleus, reflecting activation of TGF-β, Smad2 immunostains were performed on cardiac sections of hearts from fetuses dying with CHB and from a normal abortus. Smad2 nuclear translocation was observed in the 20-wk CHB and the 22-wk CHB fetuses, but not the normal 23-wk abortus (Fig. 6). Accordingly, the demonstration of Smad2 nuclear translocation

FIGURE 3. TGF-β immunoreactivity in the conduction tissue of a 22-wk fetus with CHB. A, B, and C, Longitudinal sections through the septum of a 22-wk fetus with CHB; D, a longitudinal section of septal tissue from a control 23-wk abortus. A, The section was stained with anti-TGF-β (peroxidase) and counterstained with hematoxylin. B, A neighboring region is shown after double labeling with anti-TGF-β (peroxidase) and anti-CD68 (alkaline phosphatase) to demonstrate colocalization of TGF-β and macrophages, respectively. TGF-β is present not only in the region of macrophage infiltration, but even more intensely in the adjacent zones of extensive fibrosis. C and D, The cardiac sections were double labeled with anti-TGF-β (peroxidase) and anti-SMAC (alkaline phosphatase). In the control, there was no TGF-β staining, and SMAC was only detected in blood vessel, as expected.

FIGURE 4. TGF-β immunoreactivity in the conduction tissue of a 20-wk fetus with CHB and colocalization with giant cells. Dual immunolabeling of paraffin sections from the 20-wk CHB heart establishes the spatial relationships between CD68-positive macrophages (red in A and D), SMAC-positive myofibroblasts (red in B and E), and TGF-β (brown in A, B, D, and E). A–C, Low magnification images of adjacent sections of the inflamed conduction system as it passes through the AV annulus and superior interventricular septum. Macrophages are present in the superior portion of the field in partially calcified inflamed areas (compare A and C). Myofibroblasts are concentrated in adjacent subendocardium, where the intense TGF-β immunoreactivity is also present in the surrounding fibrous matrix (compare B and C). D, Higher magnification images demonstrate colocalization of TGF-β (brown) with CD68 (red) in the cytoplasm of macrophages, including multinucleated giant cells (arrows). E, TGF-β is present in the extracellular matrix (arrowheads) surrounding myofibroblasts, but definite colocalization of SMAC and TGF-β in the latter cells is not observed. F, Inflammation, calcification, and multinucleate giant cells are concentrated in an area comparable to that shown in D.
indicates that the TGF-β present is not latent, supporting the scenario that TGF-β is secreted by infiltrating macrophages (genetically exaggerated in some fetuses) in the cardiac tissue, which in turn is activated, resulting in transdifferentiation of the fibroblasts to a scarring phenotype. Thus, TGF-β is likely to be an important fetal susceptibility factor and novel target of therapeutic strategies. Only tissue from the 20-wk fetus was available for isolation of DNA. The genotype of this fetus was TNF-α 308 G/G, TGF-β

**FIGURE 5.** TGF-β mRNA expression in conduction tissue of 20-wk fetus with CHB (A and C) and normal 23-wk abortus (B and D). A–D. Immediately adjacent to the corresponding sections in Fig. 1. Oligonucleotide in situ hybridization was performed using the sense (data not shown) and antisense TGF-β digoxigenin-labeled probes. mRNA is reported using peroxidase-conjugated anti-digoxigenin.

**FIGURE 6.** Evidence of active TGF-β in conduction tissue from two fetuses with CHB, as assessed by Smad2 nuclear translocation. A and B, Longitudinal sections through the septum of a 20-wk CHB fetus. C, Longitudinal section of septal tissue from a 22-wk CHB fetus. D, Longitudinal section of septal tissue from a healthy electively terminated 23-wk fetus. A, C, and D, The sections were stained with anti-Smad2 (peroxidase and counterstained with Congo Red). B, The section was stained with mouse IgG (isotype control). Smad2 is localized to the nucleus of the cardiac fibroblasts (myofibroblasts) in the 20- and 22-wk CHB cardiac sections. In contrast, the Smad2 is localized in the cytosolic compartment in fibroblasts of the age-matched control fetal heart. As expected, there was no uptake of primary Ab using isotype control.
codon 10 Leu/Leu, and codon 25 Arg/Arg. Notably, the genotype of an unaffected older sibling was identical, except for codon 10, which was Leu/Pro. The mother had a genotype identical with the fetus with CHB.

Discussion

Pregnant women with anti-SSA/Ro Abs, regardless of their clinical status, are at risk of having infants with CHB and/or NL rash. Retrospective and prospective clinical studies convincingly demonstrate that Abs are not the sole mediators of cardiac injury, and additional factors are required to convert risk to disease (6, 8, 27, 28). Research to date supports the likelihood that the cascade to scarring involves both proinflammatory and profibrosing components, each contributed in some measure by the fetus (9, 10, 14). The present study focused on two prototypic cytokines, TNF-α and TGF-β, integrating genetic studies on a large cohort of families with CHB and/or NL rash. TNF-α mRNA was expressed in the cardiac tissue and protein was demonstrated. An increased frequency of the −308A allele, which is associated with high cytokine production, was observed in CHB patients compared with a cohort of published Caucasian controls. However, a clear association with disease could not be established because the unaffected children and those with rash also had a higher prevalence of the −308A allele than the controls. Although TNF-α may be one of several fetal factors that amplify susceptibility, our genetic and immunohistochemical studies more convincingly suggest a link between TGF-β and the pathogenesis of CHB. TGF-β mRNA and protein were both detected in two CHB fetal hearts, and expression was particularly intense in the septal regions. Moreover, there was a significantly increased frequency of the TGF-β codon 10 fibrosis-associated polymorphism Leu10 in children with CHB compared with unaffected children.

TGF-β is a pluripotent growth factor with a central role in tissue repair and fibrosis. TGF-β activates gene transcription, thereby increasing synthesis and secretion of collagen and other matrix proteins (18). It decreases the synthesis of proteolytic enzymes, such as collagenase, which degrade matrix proteins (29). TGF-β has a complex cellular regulation that requires activation of a latent form. The active form of TGF-β is a 25-kDa disulfide-linked dimer comprised of two identical chains of 112 aa, synthesized in a latent form as a protein containing 390 aa (30). Perhaps the secretion and handling of TGF-β in the human fetal heart contribute to the increased vulnerability of this tissue to injury by maternal autoantibodies. Indeed, this could account for the absence of damage in the maternal heart. It is also intriguing that the degree of fibrosis incurred by the fetal heart may vary from one fetus to another, even in the same family. The human gene encoding TGF-β is on chromosome 19q13 and is highly polymorphic. Awad et al. (19) have identified five polymorphisms in the TGF-β gene: two in the promoter region at positions −800 and −509, one at position +72 in a nontranslated region, and two in the signal sequence at positions +869 and +915. The polymorphisms at positions +869 and +915, which change codon 10 (T or C, leucine→proline) and codon 25 (G or C, arginine→proline), respectively, are associated with individual variation in the levels of TGF-β production. This has clinical relevance because several animal and human studies have shown that high TGF-β producers develop significantly more lung fibrosis in response to a number of inflammatory triggers such as radiation (31), chemotherapy (32), and lung transplantation (33). For codon 25, the C allele encoding proline for these polymorphisms is associated with lower TGF-β synthesis in vitro and in vivo. Awad et al. (19) treated peripheral blood leukocytes with PHA and PMA and reported mean TGF-β levels of 10,037 pg/ml for Arg/Arg (n = 25) and 6,729 pg/ml for Arg/Pro (n = 9). (The authors were unable to obtain blood from a patient with the Pro/Pro genotype.) In patients requiring lung transplantation for pulmonary fibrosis, there was an increase in the frequency of the Arg25 allele (19). This allele was also associated with allograft fibrosis in transbronchial biopsies when compared with controls and with nonallograft fibrosis (34).

The literature on whether individuals with the proline allele at codon 10 produce higher or lower levels of TGF-β protein is conflicting. Perhaps the influence of the allele at codon 10 (contained within the signal sequence of the mature polypeptide) on TGF-β production is more variable due to cross-modulating interfaces between signaling networks within cells. Thus, the impact of polymorphism on phenotype may depend on the specific disease under study because of stimulus-specific contributions to signaling pathways involving translational (protein folding) and posttranslational (secretion/activation) events.

In our in vitro cardiac-injury model, we hypothesize that macrophages isolated from a Leu10/Leu10 donor secrete higher levels of TGF-β than macrophages from a Pro10/Pro10 donor after phagocytosis of opsonized apoptotic cardiocytes. The clinical significance of the polymorphism at codon 10 must also take into account the multiple biologic activities of this cytokine. It has been reported that lung allograft recipients with the leucine allele at codon 10 produced the highest amounts of TGF-β (19), and chronic rejection after lung transplant is linked with high levels of TGF-β (34). In contrast, low TGF-β production has been correlated with the chronic rejection associated with coronary artery disease after heart transplant (35). This apparent paradox can be reconciled by considering that chronic rejection after lung transplant is mediated by enhancement of extracellular matrix, while chronic rejection after heart transplant is dominated by proliferation of smooth muscle cells, which is inhibited by TGF-β (36). This latter biologic function is in keeping with the report that TGF-β levels are low in patients with advanced coronary artery disease and that high levels might protect against atherosclerosis (36). In parallel with our hypothesis that high levels of TGF-β permit the development of CHB due to enhancement of extracellular matrix and increased fibrosis, patients with cystic fibrosis who develop rapid deterioration in lung function have an increased frequency of the Leu10 homozygosity (37).

The association of TGF-β polymorphisms with increased organ fibrosis prompted the study of this cytokine in CHB. Previously, we have reported exaggerated apoptosis and its relationship to Ig deposition, macrophage infiltration, and fibroblast transdifferentiation in cardiac tissue from fetuses dying of heart block (9, 10, 14). Events subsequent to the initial inflammation triggered by opsonization of apoptotic cardiocytes may result in a critical alteration of the fibroblast phenotype that ultimately leads to fibrosis of the conducting system. An important step in defining the cross talk between these various cell types was to examine affected target tissue for the presence of regulatory molecules. The immunohistochemical sections presented in this work are the first to demonstrate significant extracellular and intracellular expression of TGF-β, although not distinguishing between active and latent forms. However, the presence of nuclear Smad2 supports that TGF-β is active. Importantly, at the atrioventricular groove, TGF-β was present in the cytoplasm of the macrophages and multinucleated giant cells. This observation supports the notion that these cells secrete TGF-β, which in turn would account for the detection of myofibroblasts in close proximity to the macrophage infiltrate.
In the conduction system and subendocardial regions, fibrous matrix containing extracellular TGF-β could be seen insinuated between the macrophage infiltrates and the more peripheral myofibroblasts. The observation that children with CHB have a higher frequency of a genetic polymorphism in TGF-β that could lead to its exaggerated secretion than unaffected anti-SSA/Ro-exposed children fits well with the histologic observations. Amplification of Ab-induced injury secondary to a genetic polymorphism that inherently leads to increased TGF-β production could be a factor relating to susceptibility. It is acknowledged that further polymorphisms need be considered, not only in the TGF-β genes, but those encoding molecules involved in the activation of latent TGF-β. Of relevance in this study may be the still unexplained clinical spectrum of disease in which some fetuses develop fibrosis in an extraordinarily short time frame, while others have incomplete blocks that progress postnataally even up to years after the Abs have been cleared from the circulation. Finally, it remains a possibility that TGF-β is not restricted to the fetal contribution, because it is known that this cytokine is readily transported across the human placenta (38). Although the Leu10 allele was not increased in the mothers, a maternal source of TGF-β remains a possibility.

The clinical and pathologic significance of the genetic predisposition to increased TNF-α production in all children of the NL families, regardless of disease status, remains unknown. Although not necessarily negating its contribution to disease susceptibility, the prevalence of the −308A polymorphism may reflect the strong linkage disequilibrium between this allele and HLA-DR3 in Caucasians (16, 39). It is also of interest that higher TNF-α serum levels have been demonstrated in Caucasians with the HLA-DR3 allele (40). Mothers of children with NL are frequently DR3, which is not surprising because this HLA-DR is strongly associated with anti-SSA/Ro-SSB/La Abs (41). Thus, it is quite likely that DR3 will also be present in many of the offspring who have the −308A allele.

Yet the possibility that fetuses whose macrophages inherently secrete amplified levels of a proinflammatory cytokine when inadvertently phagocytosing an opsonized apoptotic cardiocyte are predisposed to the development of CHB is hard to totally dismiss. Indeed, several publications support the notion that the −308A polymorphism may be biologically relevant. The haplotype containing the −308A allele has been shown in mitogen-activated PBLs and enriched monocyte cells to be associated with high TNF-α production (15). This is presumed to be due to increased binding of specific transcription factors to the area of the polymorphism. A biologic consequence of increased TNF-α secretion in our proposed genetic cascade could also be an amplification of physiologic apoptosis. Zhuang et al. (42) have reported that UVB-induced apoptosis of keratinocytes is mediated in part by TNF-α, which is secreted in response to UVB irradiation. The increased frequency of the −308A allele in the mothers would not be expected to cause cardiac problems because the cardiocytes of the adult heart are not undergoing physiologic apoptosis and therefore not expressing SSA/Ro-SSB/La on the cell surface. With regard to the children with cutaneous manifestations of NL, it is notable that patients with the photosensitive rash of subacute cutaneous lupus erythematosus have an increased prevalence of the −308A allele compared with patients who have a less photosensitive form of lupus, discoid lesions, or normal nonlupus controls (16). In fact, the name NL was originally coined based on the resemblance of the neonatal rash to subacute cutaneous lupus erythematosus and the fact that both are highly photosensitive. Our results did reveal a higher frequency of the −308A allele as well as the −308A/G genotype in children with rash compared with those with CHB or no manifestation of NL, but did not reach statistical significance, perhaps due to the smaller sample size of the former.

In summary, this translational approach evaluating genetics in the context of histology supports a new direction for further research in defining the pathogenesis of CHB. The demonstration of both TNF-α and TGF-β, albeit only protein expression of the latter, in the septic region of affected hearts provides the requisite evidence that these cytokines contribute to Ab-mediated scarring of tissue. The finding of an increased frequency of a TGF-β polymorphism (associated with organ fibrosis in other diseases) in children with CHB compared with unaffected children of anti-SSA/Ro-positive mothers is of biologic interest and complements the histologic findings. Although proof of concept awaits application to an animal model, TGF-β is likely to be an important fetal susceptibility factor, and a component of its secretion/activation may be a target of therapeutic strategies in the future.

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References

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