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Binding of Anti-SSA Antibodies to Apoptotic Fetal Cardiocytes Stimulates Urokinase Plasminogen Activator (uPA)/uPA Receptor-Dependent Activation of TGF-β and Potentiates Fibrosis

Paraskevi Briassouli,* Daniel Rifkin,† Robert M. Clancy,* and Jill P. Buyon*

In congenital heart block (CHB), binding of maternal anti-SSA/Ro Abs to fetal apoptotic cardiocytes impairs their removal by healthy cardiocytes and increases urokinase plasminogen activator (uPA)/uPA receptor (uPAR)-dependent plasmin activation. Because the uPA/uPAR system plays a role in TGF-β activation, we evaluated whether anti-Ro binding to apoptotic cardiocytes enhances plasmin-mediated activation of TGF-β, thereby promoting a profibrotic phenotype. Supernatants from cocultures of healthy cardiocytes and apoptotic cardiocytes bound by IgG from a mother whose child had CHB (apo-CHB–IgG) exhibited significantly increased levels of active TGF-β compared with supernatants from cocultures of healthy cardiocytes and apoptotic cardiocytes preincubated with IgG from a healthy donor. Treatment of the culture medium with anti-TGF-β Ab or TGF-β inhibitor (SB431542) abrogated the luciferase response, thereby confirming TGF-β dependency. Increased uPA levels and activity were present in supernatants generated from cocultures of healthy cardiocytes and apo-CHB–IgG cardiocytes compared with healthy cardiocytes and apoptotic cardiocytes preincubated with IgG from a healthy donor, respectively. Treatment of apo-CHB–IgG cardiocytes with anti-uPAR or anti-uPA Abs or plasmin inhibitor aprotinin prior to coculturing with healthy cardiocytes attenuated TGF-β activation. Supernatants derived from cocultures of healthy cardiocytes and apo-CHB–IgG cardiocytes promoted Smad2 phosphorylation and fibroblast transdifferentiation, as evidenced by increased smooth muscle actin and collagen expression, which decreased when fibroblasts were treated with supernatants from cocultures pretreated with uPAR Abs. These data suggested that binding of anti-Ro Abs to apoptotic cardiocytes triggers TGF-β activation, by virtue of increasing uPAR-dependent uPA activity, thus initiating and amplifying a cascade of events that promotes myofibroblast transdifferentiation and scar. The Journal of Immunology, 2011, 187: 5392–5401.

Organ injury induced by Abs characteristic of Sjögren’s syndrome and systemic lupus erythematosus may share in common a link between apoptosis and ultimate fibrosis (1). The signature histologic lesion of autoimmune-associated congenital heart block (CHB) is fibrosis of the atrioventricular node and, more rarely, the surrounding myocardium and endocardium (2, 3). The mechanism by which maternal anti-SSA/SSB/La Abs initiate and finally eventuate in cardiac scarring has been challenging to define, in part because the target cardiocytes are normally sequestered intracardially (1, 4). In vitro and in vivo studies suggested that apoptosis may be a key step in facilitating the accessibility of intracellular Ag to extracellular maternal autoantibodies. Previous studies using fetal cardiac myocytes demonstrated that binding of anti-SSA/Ro-SSB/La Abs to apoptotic cardiocytes impaired their removal by healthy cardiocytes and increased urokinase plasminogen activator (uPA)/uPA receptor (uPAR)-dependent plasmin activation.

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dependent activation of TGF-β is promoted by the surface localization of uPA to its receptor. Plasmin can release active TGF-β from the pentraxin complex as the result of proteolytic cleavage of the latency-associated peptide (10).

Given the significance of both plasmin and TGF-β signaling in inflammation and organ injury, we tested the hypothesis that binding of anti-SSA/Ro Abs to the surface of apoptotic cardiomyocytes leads to uPA/uPAR activation and subsequent plasmin-dependent TGF-β activation and fibrosis. This was experimentally approached using cocultures of healthy and apoptotic human fetal cardiac myocytes and autoantibodies isolated from mothers of children with CHB. Evidence of a biologic effect of TGF-β activation was sought by evaluation of Smad2 phosphorylation in separately cultured cardiac fibroblasts. Fibroblastic phenotypes were assessed by smooth muscle actin (SMAc) and collagen expression.

Materials and Methods

Reagents and commercial Abs

Pan-anti–TGF-β Ab and active and pro-anti–TGF-β were purchased from R&D Systems (Minneapolis, MN). Anti-α-SMAc and anti-tubulin Ab were from Sigma-Aldrich (St. Louis, MO). Secondary Alexa Fluor 568 was from Molecular Probes (Eugene, OR). Aprotinin was from Sigma. Anti-uPA and anti-uPAR Abs were from American Diagnostica (Stamford, CT). Anti-phospho-Smad2 Ab was from Cell Signaling (Boston, MA). Anti-Col1A mAb clone IB10 (F-4771; Sigma-Aldrich), which recognizes fibroblasts. To determine the specificity of TGF-β activation of the plasminogen activator inhibitor (PAI)-1 promoter in the TMLCs (mink lung epithelial reporter cells that stably express a portion of the PAI-1 promoter), 10 nm SB431542 (5 µmol/ml) was added to the apoptotic cells prior to incubation with the healthy cardiomyocytes. To determine the specificity of TGF-β activation of the plasminogen activator inhibitor (PAI)-1 promoter in the TMLCs (mink lung epithelial reporter cells that stably express a portion of the PAI-1 promoter), 10 nm SB431542 or 50 µg/ml Pan-anti–TGF-β Ab was added during the coculture assays. In the engulfment assays using cell-culture plates with inserts to inhibit cell contact, healthy cells were plated in the lower chamber of the culture plate, and apoptotic cardiac myocytes were added at a 2:1 ratio in the upper chamber, followed by overnight incubation.

Activation of apoptotic pathway in human fetal cardiomyocytes

For engulfment assays, healthy human fetal cardiocytes were plated in a 24-well culture plate (50,000/well) (Becton Dickinson); the next day, apoptotic cardiocytes (rendered apoptotic, as described above) were added to cultured healthy cardiomyocytes to achieve a ratio of 2:1 apoptotic cells/healthy cardiomyocytes. Before addition, the apoptotic cardiocytes, in a total volume of 1 ml DMEM plus 10% FCS, were preincubated with various Ab preparations, which included the following: CHB-IgG (0.3 mg/ml; nonopsonized), nlg-IgG (0.3 mg/ml; nonopsonized), affinity-purified anti–Ro60-IgG (0.015 mg/ml; opsonized), rabbit anti-uPA and mouse anti-uPAR (1 µg/ml; American Diagnostica); and anti-HLA (1 µg/ml; Sigma-Aldrich). To determine the contribution of plasmin to TGF-β activity, 10 µg/ml aprotinin was added to the apoptotic cells prior to incubation with the healthy cardiomyocytes. To determine the specificity of TGF-β activation of the plasminogen activator inhibitor (PAI)-1 promoter in the TMLCs (mink lung epithelial reporter cells that stably express a portion of the PAI-1 promoter), 10 nm SB431542 or 50 µg/ml Pan–anti–TGF-β Ab was added during the coculture assays. In the engulfment assays using cell-culture plates with inserts to inhibit cell contact, healthy cells were plated in the lower chamber of the culture plate, and apoptotic cardiac myocytes were added at a 2:1 ratio in the upper chamber, followed by overnight incubation.

TGF-β-activity assay

TGF-β was assayed, as described. Briefly, TMLCs were suspended at 15 × 10^5 cells/ml in DMEM containing 10% FCS. TMLCs were plated first at 100 µl per microtiter in a 96-well culture plate (Microtest III plates; Falcon, Franklin Lakes, NJ) and allowed to attach overnight. Cells were washed, and sample supernatants were added at 100 µl/well and cultured for 24 h. Luciferase activity was measured in triplicate using the Bright-Glo detection system (Promega, Madison, WI), and luminescence was determined using a Synergy 2 BioTek microplate reader (BioTek Instruments, Winooski, VT) and reported as relative light units (RLU). Intervariability in RLU measurements observed between assays was attributed to the cardiomyocytes derived from different donors.

ELISA

Total TGF-β in supernatants was measured using a TGF-β ELISA system (R&D Systems), which is designed to measure active TGF-β. Total TGF-β was assayed after acid activation by addition of 1 N HCl to samples. Total uPA was measured using a uPA ELISA system (American Diagnostica).

uPA-activity assay

uPA activation was determined using a chromogenic assay. Apoptotic cardiomyocytes were treated with CHB-IgG or nl-IgG for 30 min at room temperature (RT). In separate experiments, either anti-uPA or anti-uPAR, following preincubination with nl-IgG or CHB-IgG, or aprotinin was added during the coculture experiments. One hundred microliters of sample supernatant was added to each well, followed by the addition of microliters of human plasminogen (0.2 mg/ml in PBS) was added; after a 2-h incubation at 37°C, 15 µl chromogenic substrate for plasmin (Spectrozyme PL) was added to each well. The chromogenic substrate was

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prepared by diluting 50 μmol into 27 ml lysis buffer and then neutralizing to pH 7 with 0.6 ml 1 N HCl. The reaction product was read on a BioTek spectrophotometer (405 nm; BioTek Instruments) at 1 h, and the results were compared to a standard curve generated by serial dilutions of high molecular weight uPA.

**Immunofluorescence**

Fibroblasts were seeded on collagen-coated chamber slides and serum starved overnight before the addition of the sample supernatants. Immunofluorescence was performed on cells after fixation with 3% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) in PBS (pH 7.4) for 15 min at RT. After blocking nonspecific binding with 3% normal goat serum (Jackson ImmunoResearch, West Grove, PA), cells were incubated with the appropriate primary Abs and then with secondary Ab, anti-mouse Alexa Fluor dye 568 (Molecular Probes), at RT. Cells were stained with 2 μg/ml Hoechst 33258 for 30 min at 37°C and embedded in VECTASHIELD mounting medium (Vector Laboratories). For each sample, at least six images from three independent experiments were analyzed. Cells were viewed with an Axioplan (Carl Zeiss Meditec, Thornwood, NY) microscope, as indicated. For the Axioplan, images were captured with a charge-coupled device camera (SPOT-2; Diagnostic Instruments (Sterling Heights, MI) and processed by PhotoShop (Adobe Systems, Mountain View, CA). The images were subsequently combined and processed with ImageJ software (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij).

**Immunoblotting**

For immunoblot analysis, cells were collected from culture dishes, washed three times in PBS, and lysed in solubilization buffer (50 mmol/l HEPES [pH 7.4], buffer containing 0.1% Triton X-100, 10% glycerol, 0.5% deoxycholic acid, 150 mmol/l NaCl, 50 mmol/l NaF, 1 mmol/l NaVO₄, 100 μg/ml PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 10 μg/ml aprotinin) for 30 min at 4°C. The lysates were centrifuged at 15,000 rpm for 5 min at RT. Aliquots of supernatants were mixed with 2 μg/ml Fluor dye 568 (Molecular Probes), at RT. Cells were stained with 2 μg/ml Hoechst 33258 for 30 min at 37°C and embedded in VECTASHIELD mounting medium (Vector Laboratories). For each sample, at least six images from three independent experiments were analyzed. Cells were viewed with an Axioplan (Carl Zeiss Meditec, Thornwood, NY) microscope, as indicated. For the Axioplan, images were captured with a charge-coupled device camera (SPOT-2; Diagnostic Instruments (Sterling Heights, MI) and processed by PhotoShop (Adobe Systems, Mountain View, CA). The images were subsequently combined and processed with ImageJ software (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij).

**FIGURE 1.** CHB-IgG-treated apoptotic cardiocytes activate lTGF-β. A, TMLC-based assessment of the luciferase activity generated by incubation of apoptotic cardiocytes in the presence or absence of lTGF-β (1 ng/ml). Y-axis represents RLU, and the x-axis represents each experimental condition of apoptotic cardiocytes (either untreated or treated with nl-IgG, CHB-IgG, CHB-IgG + SB431542, or CHB-IgG + aprotinin) in the presence of 1 ng/ml of lTGF-β. B, TMLC-based assessment of the luciferase activity generated by incubation of nl-IgG, Ro60-IgG, mouse isotype, or anti-HLA–treated apoptotic cardiocytes in the presence or absence of lTGF-β (1 ng/ml). C, TMLC-based assessment of the luciferase activity generated by incubation of CHB-IgG–treated healthy cardiocytes in the presence of lTGF-β (1 ng/ml). Sample number of independent experiments is indicated, and error bars represent ± SEM. The p values < 0.05 were considered significant.

**Statistics**

For statistical analysis, the Mann–Whitney U test or the paired t test, where appropriate, was used to compare medians or means between groups in the luciferase, plasminogen, and TGF-β ELISA assays; p < 0.05 was considered statistically significant. Analyses were performed with GraphPad Prism (GraphPad Software, San Diego, CA).

**Results**

lTGF-β is activated by CHB-IgG and anti-Ro60 but not anti-HLA–treated apoptotic cardiocytes

We initially addressed whether the increased plasmin generated by uPA/uPAR induced by anti-SSA/Ro 60 binding to apoptotic cardiocytes resulted in increased activation of exogenously provided lTGF-β. Cultured cardiac myocytes were rendered apoptotic by exposure to staurosporine, and apoptosis was confirmed by microscopic observation of cell size and morphology, as well as flow cytometric analysis of phosphatidylserine exposure by binding of Annexin V-FITC. The addition of lTGF-β (1 ng/ml) to apoptotic cardiocytes treated with CHB-IgG, but not nl-IgG, increased luciferase activation of a mink epithelial TGF-β reporter cell line (TMLC) (1021 ± 192 CHB-IgG + lTGF-β RLU versus 496 ± 131 nl-IgG + lTGF-β RLU; p = 0.008; n = 5) (Fig. 1A). This activity was diminished to baseline levels following addition of the TGF-βR kinase inhibitor (SB431542) (1021 ± 192 CHB-IgG + lTGF-β RLU versus 496 ± 131 nl-IgG + lTGF-β RLU; p = 0.008; n = 5) (Fig. 1A). This activity was diminished to baseline levels following addition of the TGF-βR kinase inhibitor (SB431542) (1021 ± 192 CHB-IgG + lTGF-β RLU versus 496 ± 131 nl-IgG + lTGF-β RLU; p = 0.008; n = 5) (Fig. 1A). This activity was diminished to baseline levels following addition of the TGF-βR kinase inhibitor (SB431542)}
apoptotic cardiocytes were the source of \( \text{lTGF-\beta} \) addressed whether healthy cardiocytes undergoing efferocytosis of treated cardiocytes activated exogenously added \( \text{lTGF-\beta} \). Having established that apoptotic-CHB–IgG (apo-CHB–IgG)–myocytes with CHB-IgG–bound apoptotic cardiac myocytes enhance activation of TGF-\( \beta \) does not bind healthy cells (6). An additional control included incubation of apoptotic cardiocytes with anti-HLA Abs, which also bind the surface of apoptotic cardiocytes. This condition did not activate \( \text{lTGF-\beta} \) (1740 ± 350 Ro60-IgG RLU versus 350 ± 11 anti-HLA RLU; \( p = 0.05; n = 3 \)) (Fig. 1B). In contrast to apoptotic cells, healthy cardiocytes treated with either CHB-IgG or nl-IgG did not activate \( \text{lTGF-\beta} \) (514 ± 108 CHB-IgG RLU versus 524 ± 119 nl-IgG RLU; \( p = 0.6; n = 3 \)) (Fig. 1C), indicating a requirement for surface-exposed Ro60 on the apoptotic cells, because CHB-IgG does not bind healthy cells (6).

Enhanced activation of TGF-\( \beta \) in cocultures of healthy cardiac myocytes with CHB-IgG–bound apoptotic cardiac myocytes

Having established that apoptotic-CHB–IgG–cardiocytes (apo-CHB–IgG)–treated cardiocytes activated exogenously added \( \text{lTGF-\beta} \), we next addressed whether healthy cardiocytes undergoing efferocytosis of apoptotic cardiocytes were the source of \( \text{lTGF-\beta} \). Initial experiments demonstrated that efferocytosis of apoptotic cardiocytes by healthy cardiocytes resulted in enhanced activation of TGF-\( \beta \), albeit at modest levels (158 ± 14 healthy cardiocytes RLU versus 328 ± 12 healthy + apoptotic cardiocytes RLU; \( p = 0.04; n = 3 \)) (Fig. 2A). Because the binding of CHB-IgG to apoptotic cardiocytes was the generation of plasmin (5), we investigated whether the addition of apo-CHB–IgG cardiocytes to healthy cardiocytes resulted in TGF-\( \beta \) activation similar to that observed when \( \text{lTGF-\beta} \) was exogenously added (Fig. 1A). Supernatants generated following an overnight incubation of healthy cardiocytes and apo-CHB–IgG cardiocytes exhibited a significant increase in luciferase activation compared with supernatants generated following incubation of healthy cardiocytes with apo-nl–IgG cardiocytes (nl-IgG–treated) (511 ± 204 CHB-IgG RLU versus 156 ± 121 nl-IgG RLU; \( p = 0.002; n = 7 \)) or compared with supernatants from healthy cardiocytes alone (Fig. 2B) (511 ± 204 CHB-IgG RLU versus 103 ± 39 healthy cardiocytes RLU; \( p = 0.03; n = 7 \)). Supernatants from apo-nl–IgG or apo-CHB–IgG cardiocytes showed no increase in luciferase activation over background (511 ± 204 apo-CHB–IgG from cocultures with healthy cardiocytes RLU versus 280 ± 85 apo-CHB–IgG alone RLU; \( p = 0.002; n = 7 \)) (Fig. 2B).

Similar results were obtained from cocultures of apoptotic cardiocytes opsonized with three other CHB-IgG sera (CHB1, CHB2, CHB3): 780 ± 94 CHB1-IgG RLU versus 340 ± 40 nl-IgG RLU; \( p = 0.02; n = 3 \); 914 ± 147 CHB2-IgG RLU versus 340 ± 40 nl-IgG RLU; \( p = 0.01; n = 3 \); and 725 ± 136 CHB3-IgG RLU versus 371 ± 96 nl-IgG RLU; \( p = 0.008; n = 5 \), indicating that the luciferase activation was specific to TGF-\( \beta \).

\[ \text{FIGURE 2.} \]

Cocultures of healthy cardiocytes with CHB-IgG–bound apoptotic cardiocytes activate TGF-\( \beta \). A, TMLC-based assessment of the luciferase activity generated by cocultures of healthy cardiocytes with apoptotic cardiocytes or apoptotic cardiocytes alone. X-axis represents RLU. B, TMLC-based assessment of the luciferase activity generated by cocultures of healthy cardiocytes with apo-nl–IgG or apo-CHB–IgG cardiocytes or Ab-treated cardiocytes alone. C, TMLC-based assessments of the luciferase activity generated by cocultures of healthy cardiocytes with apo-nl–IgG or apo-CHB–IgG cardiocytes or Ab-treated cardiocytes treated with either nl-IgG or different IgG fractions CHB-IgG sera (CHB1, CHB2, CHB3). D, Inhibition of TGF-\( \beta \) luciferase activation of TMLCs exposed to supernatants of cocultures of healthy cardiocytes with apo-CHB–IgG cardiocytes in the presence of either Pan–anti–TGF-\( \beta \) Ab or small-molecule TGF-\( \beta \) inhibitor (SB431542). Sample number of independent experiments is indicated, and error bars represent ± SEM. The \( p \) values < 0.05 were considered significant.
Previously shown (5), enzymatic activity was decreased when apo-ChB–IgG cardiocytes were subsequently treated with Abs against either uPA (5.3 ± 1.1 CHB-IgG U/ml versus 2 ± 0.2 CHB-IgG + anti-uPAR U/ml; p = 0.04; n = 3) or uPA (5.3 ± 1.1 CHB-IgG U/ml versus 1.9 ± 0.7 CHB-IgG + anti-uPA U/ml; p = 0.05; n = 3) or when aprotinin was present (5.3 ± 1.1 CHB-IgG U/ml versus 1.5 ± 0.6 CHB-IgG + aprotinin U/ml; p = 0.05; n = 3) (Fig. 3A). The effect of these various inhibitory Abs on TGF-β activation was assessed by incubating the TMLCs with the supernatants from the efferocytosis assays. TGF-β activation was attenuated when plasminogen activation was suppressed by anti-uPA or anti-uPAR Abs or by aprotinin: 805 ± 57 CHB-IgG RLU versus 197 ± 56 CHB-IgG + anti-uPAR RLU; p = 0.002; n = 3; 805 ± 57 CHB-IgG RLU versus 381 ± 123 CHB-IgG + anti-uPA RLU; p = 0.05; n = 3; and 805 ± 57 CHB-IgG RLU versus 385 ± 52 CHB-IgG + aprotinin RLU; p = 0.007; n = 3, respectively (Fig. 3B).

The source of plasmin mediating the anti-TGF-β activation was determined. Coculture assays of healthy and apoptotic cardiocytes were conducted in culture media containing serum devoid of plasminogen. The absence of plasminogen resulted in concomitant loss of CHB-IgG–dependent TGF-β luciferase activation (933 ± 42 CHB-IgG with plasminogen RLU versus 325 ± 76 CHB-IgG without plasminogen RLU; p = 0.007; n = 3) and (170 ± 72 healthy cardiocytes without plasminogen RLU versus 325 ± 76 CHB-IgG without plasminogen RLU; p = 0.2; n = 3) (Fig. 3C).

FIGURE 3. Anti-Ro–dependent uPA/uPAR plasminogen activation is responsible for the generation of active TGF-β. A, A chromogenic enzymatic activity assay was used to evaluate plasminogen activation of supernatants generated from cocultures of healthy cardiocytes with apo-CHB–IgG cardiocytes or apo-CHB–IgG cardiocytes subsequently treated with Abs against uPA or uPAR or incubated in the presence of aprotinin (10 μg/ml). B, Inhibition of TGF-β–mediated luciferase activation of TMLCs exposed to supernatants of healthy cardiocytes with apo-CHB–IgG cardiocytes or apo-CHB–IgG cardiocytes subsequently treated with Abs against uPA or uPAR or incubated in the presence of aprotinin (10 μg/ml). C, Contribution of plasminogen to the CHB-IgG–mediated TGF-β luciferase activation. Supernatants of cocultures of healthy cardiocytes with apo-nl–IgG or apo-CHB–IgG cardiocytes, conducted in the presence or absence of plasminogen-containing media, were added to the TMLCs; luciferase activity was determined after 24 h of incubation. D, Contribution of cell contact in CHB-IgG–mediated TGF-β luciferase activation. Healthy cardiocytes were cocultured with apo-nl–IgG or apo-CHB–IgG cardiocytes either together or separated by cell-culture plate inserts to inhibit direct cell contact. After overnight incubation, supernatants were collected and added to TMLCs. Y-axis represents RLU. Sample number of independent experiments is indicated; error bars represent ± SEM. The p values < 0.05 were considered significant.
We next examined the necessity for direct cell contact between the healthy cardiocytes and the apoptotic cardiocytes to trigger TGF-β activation. Coculture assays were performed in which a cell-culture plate insert was introduced to separate the healthy cells from the apoptotic cells, while allowing soluble molecules to pass through. Interference with cell contact between the healthy and apoptotic cardiocytes resulted in decreased CHB-IgG-dependent TGF-β activation (794 ± 85 CHB-IgG RLU versus 305 ± 117 CHB-IgG no contact RLU; p = 0.01; n = 3) (Fig. 3D).

**Total levels of TGF-β are decreased, but uPA levels are elevated, in cocultures of CHB-IgG–bound apoptotic cardiomyocytes and healthy cardiocytes**

The total (active plus latent) level of TGF-β was evaluated to determine whether the observed increased TGF-β activation in cocultures of apo-CHB–IgG cardiocytes with healthy cardiocytes was due to increased TGF-β protein secretion. Total TGF-β levels were significantly decreased in the supernatants from cocultures of healthy cardiocytes and apo-CHB–IgG cardiocytes compared with apo-nl–IgG cardiocytes (2684 ± 530 pg/ml nl-IgG versus 1183 ± 243 pg/ml CHB-IgG; p = 0.002; n = 10), as assessed by ELISA (Fig. 4A). These data are consistent with the previous observation that the binding of CHB-IgG to apoptotic cardiocytes decreased their effectorcytosis by healthy cardiocytes. These same supernatants contained statistically significant levels of uPA protein (999 ± 830 pg/ml CHB-IgG versus 536 ± 436 pg/ml nl-IgG; p = 0.03; n = 10), suggesting that, despite the decreased levels of total TGF-β, increased uPA-dependent enzymatic activity results in increased TGF-β activation (Fig. 4B).

**CHB-IgG–dependent TGF-β activation promotes a scarring phenotype in the cardiac fibroblasts**

TGF-β elicits its biologic effects by interacting with TGF-βRI/II receptors, which result in Smad2 phosphorylation and its subsequent nuclear translocation and transactivation of gene expression (12). Evidence substantiating an effect of active TGF-β on the cardiac fibroblast was sought by evaluating Smad2 phosphorylation. Elevated phospho-Smad2 was detected when fibroblasts were treated with supernatants of cocultures of healthy cardiomyocytes with apo-CHB–IgG cardiocytes, similar to that observed when fibroblasts were directly treated with rTGF-β (Fig. 5).

The effect of CHB-IgG–mediated TGF-β activation was evaluated for its ability to promote fibrosis on fetal cardiac fibroblasts. We monitored levels of SMAC as a read-out of fibroblast trans-differentiation. Fibroblasts were serum starved overnight and subsequently treated with supernatants from cocultures of healthy cardiomyocytes incubated with apo-CHB–IgG or apo-nl–IgG cardiocytes. Increased SMAC expression was observed when fibroblasts were incubated with supernatants derived from cocultures of healthy cardiomyocytes with apo-CHB–IgG cardiocytes (Fig. 6F). Addition of recombinant active TGF-β served as a positive control (Fig. 6A). Attenuation of SMAC expression was observed when fibroblasts were treated with supernatants generated from cocultures of healthy cardiomyocytes with apo-CHB–IgG cardiocytes subsequently treated with anti-uPAR Abs (Fig. 6F) or SB43152 (Fig. 6I). The results obtained by immunofluorescence were paralleled by immunoblot of the fibroblast lysates treated under identical conditions. As illustrated in Fig. 7 (lanes 3 and 9), SMAC protein expression was increased in fibroblasts treated with supernatants from cocultures of healthy cardiomyocytes and apo-CHB–IgG cardiocytes.

As an additional read-out of fibroblast trans-differentiation, collagen expression was assessed in fibroblasts treated with supernatants from cocultures of healthy cells and apoptotic cells treated with the various Ab conditions, as above. Increased collagen expression was noted when fibroblasts were incubated overnight with supernatants of healthy cardiomyocytes with apo-CHB–IgG cardiocytes (Fig. 8F).

**Discussion**

Identification of the molecular components that contribute to the crosstalk between inflammation and fibrosis is an important step in linking anti-SSA/Ro Abs to cardiac injury. An abundance of apoptotic cardiomyocytes is a consistent histologic finding in hearts of fetuses dying shortly after the diagnosis of CHB (8). By applying this clue to an in vitro culture system of human fetal cardiomyocytes, we recently demonstrated that the binding of anti-SSA/Ro Abs resulted in attenuation of effectorcytosis by healthy cardiomyocytes (supporting the exaggerated apoptosis), a modification of uPAR expression and uPA activation and, ultimately, the generation of plasmin (5, 6). Our studies identified a functional and potentially pathologic consequence of the latter enzymatic activity. Apoptotic anti-SSA/Ro–bound cardiomyocytes mediated increased activation of exogenously added lTGF-β compared with control Ab-treated apoptotic cardiomyocytes. Activation of lTGF-β secreted during effectorcytosis of apoptotic cardiomyocytes by healthy cardiomyocytes was significantly increased in supernatants from cocultures containing anti-SSA/Ro–bound apoptotic cardiomyocytes compared with
cocultures with unopsonized apoptotic cardiocytes. TGF-β activation was dependent on contact between the apoptotic cardiocyte and healthy cardiocyte rather than engulfment per se. The requirement for uPA activity was supported by the decrease in TGF-β activation observed when the opsonized apoptotic cardiocytes were treated with anti-uPAR or anti-uPA Abs or the plasmin inhibitor aprotinin prior to coculturing with healthy cardiac myocytes. The addition of supernatants derived from cocultures of healthy cardiac myocytes and opsonized apoptotic cardiocytes to cardiac fibroblasts supported the biologic activity of TGF-β, as evidenced by Smad2 phosphorylation. The profibrotic consequence of TGF-β activation generated under these conditions was illustrated by myofibroblast transdifferentiation (SMAc staining) of and increased collagen protein expression in the cardiac fibroblasts.

Although TGF-β is important in regulating crucial cellular activities, the full spectrum of molecular mechanisms that promote its activation still needs to be delineated. Some of the known activating pathways are cell or tissue specific, whereas others are common to multiple cell types and tissues. Proteases, integrins, pH, and reactive oxygen species are among the currently identified factors that can activate TGF-β (10). Plasmin was the first

![FIGURE 5](http://www.jimmunol.org/DownloadedFrom)

**FIGURE 5.** Supernatants from cocultures of healthy cardiocytes and CHB-IgG–bound apoptotic cardiocytes trigger activation of TGF-β pathway, as depicted by increased phospho-Smad2 phosphorylation. Fibroblasts were prepared as monolayers and seeded on collagen-treated culture slides. Following overnight serum starvation, cells were treated with supernatants from healthy cardiac culture supernatants from cocultures of healthy cardiocytes cultures, cocultures of healthy cardiocytes with anti-uPAR–treated apoptotic cardiocytes, cocultures of healthy cardiocytes with apo-nl–IgG cardiocytes, cocultures of healthy cardiocytes with apo-CHB–IgG cardiocytes, cocultures of healthy cardiocytes with initially apo-nl–IgG and then anti-uPAR cardiocytes, cocultures of healthy cardiocytes with initially apo-CHB–IgG and then anti-uPAR–treated cardiocytes cocultures of healthy cardiocytes with mouse anti-uPAR–treated apoptotic cardiocytes, or cocultures of healthy cardiocytes with apo-CHB–IgG cardiocytes in the presence of SB431542 or were treated with rTGF-β (100 pg/ml). Cell lysates were collected and subjected to electrophoresis, followed by immunoblotting for anti-phospho-Smad2. Equal loading was confirmed with anti-tubulin.

![FIGURE 6](http://www.jimmunol.org/DownloadedFrom)

**FIGURE 6.** Supernatants from cocultures of healthy cardiocytes and CHB-IgG–bound apoptotic cardiocytes promote transdifferentiation of human fetal cardiac fibroblasts, as depicted by increased SMAc staining. Fibroblasts were prepared as monolayers and seeded on collagen-treated culture slides. Following overnight serum starvation, cells were treated with rTGF-β (100 pg/ml) (A) or exposed to supernatants from healthy cardiac cultures (B), supernatants from cocultures of healthy cardiocytes with isotype control-treated apoptotic cardiocytes (C), cocultures of healthy cardiocytes with anti-uPAR–treated apoptotic cardiocytes (D), cocultures of healthy cardiocytes with apo-nl–IgG cardiocytes (E), cocultures of healthy cardiocytes with apo-CHB–IgG cardiocytes (F), cocultures of healthy cardiocytes with initially apo-nl–IgG and then anti-uPAR cardiocytes (G), cocultures of healthy cardiocytes with initially apo-CHB–IgG and then anti-uPAR–treated cardiocytes cocultures of healthy cardiocytes with mouse anti-uPAR–treated apoptotic cardiocytes (H), or cocultures of healthy cardiocytes with apo-CHB–IgG cardiocytes in the presence of SB431542 (I). Fibroblasts were then fixed, stained, and analyzed by fluorescence microscopy (original magnification ×40). Results are representative of three experiments.
protease documented to have TGF-β-activating capacity, and it was hypothesized that plasmin-mediated proteolysis of thrombus-associated TGF-β may act as a slow-release mechanism for TGF-β following acute injury.

Plasmin has long been considered a protease with fibrinolytic effects; however, plasmin is involved in a plethora of cellular processes through its cleavage and activation of nonfibrin substrates in the extracellular matrix. Such substrates include matrix metalloproteinases, TGF-β, and other growth factors, which can be liberated, thereby inducing signaling changes in the resident tissue microenvironment. Supportive evidence for a profibrotic role of plasmin has been provided in several experimental systems. In an experimental model of progressive kidney disease, using $\text{Plg}^{-/-}$ and $\text{Plg}^{+/+}$ mice, Ghosh et al. (13) demonstrated that the presence of plasmin is necessary to promote fibrosis through epithelial to mesenchymal transition and activation of TGF-β and PAR-1/ERK-signaling pathways. Compared with $\text{Plg}^{+/+}$ mice, in which fibrosis progresses over time, $\text{Plg}^{-/-}$ mice develop fibrosis at a significantly slower pace, which temporally regresses. In murine embryonic PAI-knockout fibroblasts, plasmin inhibition attenuates

**FIGURE 7.** Supernatants from cocultures of healthy cardiocytes and CHB-IgG-bound apoptotic cardiocytes promote fibroblast transdifferentiation, as demonstrated by increased SMAc expression. Fibroblasts were prepared as monolayers and seeded on collagen-treated culture slides. Following overnight serum starvation, cells were treated with supernatants from healthy cardiocyte cultures, supernatants from cocultures of healthy cardiocytes with isotype control-treated apoptotic cardiocytes, cocultures of healthy cardiocytes with anti-uPAR–treated apoptotic cardiocytes, cocultures of healthy cardiocytes with apo-nl–IgG cardiocytes, cocultures of healthy cardiocytes with apo-CHB–IgG cardiocytes, cocultures of healthy cardiocytes with initially nl-IgG and then anti-uPAR–treated apoptotic cardiocytes, cocultures of healthy cardiocytes with initially apo-CHB–IgG and then anti-uPAR–treated cardiocytes cocultures of healthy cardiocytes with mouse anti-uPAR–treated apoptotic cardiocytes, or cocultures of healthy cardiocytes with apo-CHB–IgG cardiocytes in the presence of SB431542 or were treated with rTGF-β (100 pg/ml). Cell lysates were collected and subjected to electrophoresis, followed by immunoblotting for anti-SMAc. Equal loading was confirmed with anti-tubulin.

**FIGURE 8.** Supernatants from cocultures of healthy cardiocytes and CHB-IgG–bound apoptotic cardiocytes promote transdifferentiation of human fetal cardiac fibroblasts, as depicted with increased collagen (Col1A) staining. Fibroblasts were prepared as monolayers and seeded on collagen-treated culture slides. Following overnight serum starvation, cells were treated with rTGF-β (100 pg/ml) (A) or exposed to supernatants from healthy cardiocyte cultures (B), supernatants from cocultures of healthy cardiocytes with isotype control treated-apoptotic cardiocytes (C), cocultures of healthy cardiocytes with anti-uPAR–treated apoptotic cardiocytes (D), cocultures of healthy cardiocytes with apo-nl–IgG cardiocytes (E), cocultures of healthy cardiocytes with apo-CHB–IgG cardiocytes (F), cocultures of healthy cardiocytes with initially apo-nl–IgG and then anti-uPAR cardiocytes (G), cocultures of healthy cardiocytes with initially apo-CHB–IgG and then anti-uPAR cardiocytes cocultures of healthy cardiocytes with mouse anti-uPAR–treated apoptotic cardiocytes (H), or cocultures of healthy cardiocytes with apo-CHB–IgG cardiocytes in the presence of SB431542 (I). Fibroblasts were then fixed, stained, and analyzed by fluorescence microscopy. Original magnification $\times 40$. Results are representative of three experiments.
TGF-β activation and subsequent fibrosis (14). Additionally, in a murine model of PAI-1 deficiency, increased plasmin enzymatic activity and TGF-β signaling contribute to cardiac fibrosis (13, 14). Specific to the relationship between organ injury and fibrosis observed in the pathology of CHB, a relative absence of TGF-β has been implicated as a major cause of scarless repair. In another murine model, developmentally early-stage fetal fibroblasts show decreased plasmin and subsequent TGF-β activity compared with later-stage or adult fibroblasts, which contract and repair wounds in a plasmin–TGF-β–dependent manner (15). These findings support a dual role for plasmin in the balance between TGF-β–mediated fibrosis-fibrinolysis and inflammation.

Our initial experiments showed that apo-CHB-IgG cardiocytes were capable of activating exogenously provided TGF-β compared with control apo-nl-IgG cardiocytes. Binding of the apoptotic surface by Ro60-IgG, but not anti-HLA, surface binding resulted in TGF-β activation. This observation suggested that the effect of anti-Ro60 is specific and not simply the result of Ab binding to cognate surface-exposed Ag. Similar effects were obtained using supernatants derived from cocultures of anti-Ro–bound apoptotic cardiocytes and healthy cardiocytes (source of ltTGF-β), in which activation of TGF-β was significantly greater compared with supernatants generated from healthy cardiocytes that efficiently engulfed the unopsonized apoptotic cardiocytes. Minimal activation of TGF-β was observed in the case of apoptotic cells alone, most likely attributed to the TGF-β present in the serum of the culture medium. Furthermore, blocking plasmin generation by either anti-uPA or anti-uPAR Abs or protease inhibition led to ablation of the TGF-β activation, supporting our hypothesis that the TGF-β activation was attributed to a CHB-dependent uPA/uPAR-mediated plasminogen activation. The source of plasminogen was shown to be the serum present in culture media (presumably in vivo, plasminogen is present in the fetal circulation), because no TGF-β activation was observed during efferocytosis when serum depleted of plasminogen was used.

Cell contact was necessary for significant TGF-β activation. This was expected because these enzymatic-activation reactions are more efficient when focused on the cell surface. For example, receptor-anchored plasmin generation on the cell surface is substantially greater than that occurring in solution. Thus, cell-surface generation of active plasmin on the opsonized apoptotic cardiocyte adjacent to the TGF-β secreted by the resident healthy cell results in enhanced efficient activation. Furthermore, mannos–6-phosphate/insulin–like growth factor II-receptor, a receptor known to bind ltTGF-β (16), interacts with uPAR on the surface of human monocytes and directly binds plasminogen. Conversion to plasmin within the complex mediates the release of active TGF-β. Thus, a similar mechanism might occur in the coculture system, wherein the increased uPA at the surface of the CHB-IgG opsonized apoptotic cardiocytes interacts with the ltTGF-β/Man–6-phosphate/insulin–like growth factor II-receptor complex on the surface of the healthy cell. Engulfment is not required for this process. Accordingly, impaired clearance of anti-Ro–bound apoptotic cardiocytes by healthy cardiocytes and the consequent persistence of CHB-IgG–bound apoptotic cardiocytes may promote continued conversion of ltTGF-β to active TGF-β.

Although the activation of TGF-β is significantly greater when apo-CHB–IgG cardiocytes are cocultured with healthy cells compared with unopsonized apoptotic cardiocytes, the levels of total TGF-β in the conditioned medium are decreased. This observation is consistent with the decreased efferocytosis observed with cocultures of apo-CHB–IgG cardiocytes, assuming the generation of total TGF-β by healthy cardiocytes is dependent on efficient engulfment. Because ltTGF-β is generally in excess, and only a small percentage of the total is activated, increased protease activity in the presence of anti-Ro Abs, combined with minimal efferocytosis, would be sufficient to lead to significantly more active TGF-β compared with control conditions. Increased uPA levels and activity were found in the presence of anti-Ro Abs, supporting the protease-dependent activation of TGF-β by CHB-IgG–generated plasmin.

Functional support for a profibrotic effect of increased TGF-β activation in the pathologic development of CHB was obtained. Increased SMAc and collagen staining, consistent with transdifferentiation of fibroblasts to myofibroblasts, was observed when fibroblasts were treated with supernatants generated from cocultures of healthy cardiocytes and opsonized apoptotic cardiocytes. This effect was abrogated when uPAR was blocked, exemplifying the tight relationship between plasmin generation and TGF-β–signaling pathways. The contribution of macrophages, which are present in the majority of autopsy specimens of affected fetal hearts, may result in an amplification of this profibrotic process. Precedent for this is the observation that supernatants generated by coculturing macrophages with opsonized apoptotic cardiocytes also transdifferentiate cardiac fibroblasts (7). The observed up-regulation of SMAc in the presence of healthy cardiocytes and opsonized apoptotic cardiocytes absent macrophages may serve as a minimal threshold to tip the cascade of signaling toward a profibrogenic phenotype.

Fibrosis of the atrioventricular node and endocardium may reflect a perilous synergy between two ongoing attempts to clear apoptotic cardiocytes: one innate process consisting of resident healthy cardiocytes, whereas the other process is adaptive, represented by recruited professional scavengers, such as macrophages. A key role for uPA-secreting macrophages in the development of cardiac fibrosis was shown in a mouse model in which recipients of bone marrow transplants from uPA-overexpressing donors, but not nontransgenic donors, developed cardiac macrophage accumulation and fibrosis (17, 18).

Collectively, our data further define the pathologic consequences initiated when maternal anti-Ro Abs bind cognate Ag on the surface of apoptotic fetal cardiocytes. Increased uPA-dependent uPA activity triggers TGF-β activation, thus initiating and amplifying a cascade of events that promotes myofibroblast transdifferentiation and scar. These observations provide rationale for therapeutic strategies aimed at attenuating plasmin-mediated TGF-β activation.

Disclosures

The authors have no financial conflicts of interest.

References


