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Transdifferentiation of Cardiac Fibroblasts, a Fetal Factor in Anti-SSA/Ro-SSB/La Antibody-Mediated Congenital Heart Block


The signature lesion of autoantibody-associated congenital heart block (CHB) is fibrosis of the conducting tissue. To date, participation of myofibroblasts in the cascade to injury has been unexplored. The importance of myofibroblast/macrophase cross-talk is demonstrated by the novel finding of these cell types in the heart of a neonate dying of CHB. This clue to pathogenesis prompted consideration of the mechanism by which maternal anti-SSA/Ro-SSB/La Abs initiate an inflammatory response and promote fibrosis. Isolated cardiocytes from 16–24 wk abortuses were rendered apoptotic by exposure to poly (2-) hydroxethylmethacrylate; flow cytometry confirmed surface expression of Ro/La. Apoptotic cardiocytes were incubated with affinity-purified Abs to 52 and 60 kDa Ro from CHB mothers (opsonized) or IgG fractions from healthy donors (nonopsonized). Macrophages cultured with opsonized apoptotic cardiocytes expressed proinflammatory markers, supported by a three-fold increase in active $\alpha$,$\beta$ integrin. Fetal cardiac fibroblasts exposed to supernatants obtained from macrophages incubated with opsonized apoptotic cardiocytes (but not nonopsonized) dramatically increased expression of the myofibroblast marker $\alpha$-smooth muscle actin (SMAc). The “opsonized” supernatant reversed an inhibitory effect of the “nonopsonized” supernatant on proliferation of fibroblasts (120 vs 69%, $p < 0.05$). Parallel experiments examined the effects of two cytokines and their neutralizing Abs on fibroblasts. TGF$\beta$1 increased SMAc staining but decreased proliferation. TNF-\alpha did not affect either readout. Addition of anti-TGF$\beta$1 Abs to the “opsonized” supernatant blocked SMAc expression but increased proliferation, while anti-TNF-\alpha blocking Abs had no effects. These data suggest that transdifferentiation of cardiac fibroblasts to a scarring phenotype is a pathologic process initiated by maternal Abs. The Journal of Immunology, 2002, 169: 2156–2163.

A role for anti-SSA/Ro-SSB/La Abs in the pathogenesis of congenital heart block (CHB) is supported by the nearly universal finding of these autoantibodies in mothers of affected children (1), and by a recently established murine model of CHB in which the offspring of mice immunized with the candidate Ags developed CHB (2). Despite advances in molecularly defining the target Ags, the how and why of Ab-mediated insult still remain largely unanswered. One major problem is finding a reasonable explanation for accessibility of these intracellular Ags to extracellular maternal Abs. Evidence is emerging to support a role for physiologic apoptosis as a mechanism of translocating these Ags to the cell surface, where they can be bound by cognate maternal Abs and inadvertently program an inflammatory response by the macrophages (3, 4).

Subsequent events initiated by the candidate maternal autoantibodies likely result in scarring, given that fibrosis of the atrioventricular (AV) node is the characteristic histopathologic lesion of CHB. Ho et al. (5) reported replacement of the AV node by fibrosis in seven hearts with CHB in association with maternal SSA/Ro Abs. The response of the fetal cardiac fibroblast may be a critical factor contributing to the ultimate expression of disease. Under physiologic conditions, healing of a wound is a well-orchestrated phenomenon involving a sequence of events in which the ability to lay down granulation tissue depends on a proliferative phase of “repair” cells and a remodeling phase to achieve a maturation of the connective tissue matrix. The transdifferentiation of a fibroblast to a myofibroblast is recognized as a pivotal component of granulation tissue formation (6). However, there is evidence that scarring results from disruption of the healing process due to the continued presence of myofibroblasts. For example, myofibroblasts persist in hypertrophic scars and in fibrotic lesions of many organs such as liver cirrhosis, lung fibrosis, kidney fibrosis (the mesangium of experimental glomerulonephritis), stromal reaction to epithelial tumors, and cardiac fibrosis (6).

Because the signature lesion of CHB is fibrosis of the AV node, the present study was initiated to determine the relationship between early Ab-mediated inflammatory events and the final sequelae leading to fibrosis. It is hypothesized that CHB is a consequence of unresolved scarring of the AV node secondary to the transdifferentiation of cardiac fibroblasts to unchecked proliferating myofibroblasts, a pathologic process initiated by maternal autoantibodies. To address this hypothesis, we extensively defined the histopathology of a heart obtained from a neonate who died of CHB (7). In vitro studies were performed to recapitulate the cascade leading to fibrosis. Separate cultures of myocytes and fibroblasts isolated from human fetal hearts were established. The cardiac myocytes were rendered apoptotic and subsequently treated with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 Abbreviations used in this paper: CHB, congenital heart block; AV, atrioventricular; SMAc, $\alpha$-smooth muscle actin; polyHEMA, poly (2-) hydroxethylmethacrylate.

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with IgG fractions from a healthy donor, a patient with systemic lupus erythematosus absent anti-SSA/Ro-SSB/La Abs, or a mother with anti-SSA/Ro-SSB/La Abs whose child had cutaneous neonatal lupus, or with Abs to components of SSA/Ro affinity-purified from a mother whose child has CHB, and incubated with macrophages isolated from the PBMC of healthy controls. The supernatants generated under these varied conditions were tested for their effects on the cardiac fibroblasts. To confirm activation of the fibroblasts, evidence was sought for transdifferentiation to a myo-fibroblast phenotype (assessed by expression of α-smooth muscle actin (SMAc)) and for proliferation (assessed by incorporation of tritiated thymidine). Molecular characterization of the effects on fibroblast phenotype was assessed by the direct addition of two candidate cytokines, TNF-α and TGFβ, as well as supernatants (generated by the macrophages) that were preincubated with TNF-α- and TGFβ-neutralizing mAbs.

Materials and Methods

Immunostaining of heart from autopsy study of neonate with CHB

Formalin-fixed paraffin sections were obtained from hearts of an infant diagnosed in utero with CHB and dying at birth (7), a human fetus electively aborted at 24 gestational weeks, and a term newborn dying of non-cardiac causes. Sections were immunostained with primary mAbs: anti-SMAc (used at 1/200; DAKO, Carpinteria, CA), which reacts with smooth muscle cells lining blood vessels and myofibroblasts, and anti-CD68 (1/3200) (Accurate Chemical and Scientific, Westbury, NY), which stains macrophages. Sections were visualized using the Vectastain avidin-biotin-peroxidase detection system (Vector Laboratories, Burlingame, CA), and counterstained with hematoxylin before photography.

Isolation and culture of cardiac myocytes and fibroblasts from human fetal tissue

Human fetal cardiocytes and human fetal fibroblasts were cultured as described (8). Briefly, human fetal hearts of gestational age 16–24 wk were aseptically obtained after elective termination of normal pregnancy by dilatation and evacuation. This was done in accordance with the guidelines of the Institutional Review Board and after obtaining consent from the mothers. The aorta was cannulated for continuous perfusion of the coronary arteries using a Langendorff preparation (9). The heart was treated with collagenase A (type 111), which was recirculated for ~20 min. The heart dissociated spontaneously, allowing cells to slowly drip and fall on a Petri dish containing 0.25% trypsin, 1 mM EDTA in HBSS. Clumps of cells were dissociated and the resulting suspension was poured over a cell strainer. Cells were centrifuged and the pellet was resuspended in 20 ml of culture medium (DMEM supplemented with 10% FBS, 50 U/ml penicillin, 50 U/ml streptomycin, 100 mg/ml gentamicin, 1 mM nonessential amino acid (Life Technologies, Rockville, MD), 0.1 mM essential medium vitamins (Life Technologies), 2 mM glutamine, 0.1 mM Na pyruvate).

The cell isolate contained both cardiac myocytes and fibroblasts. Separate enriched cultures of each cell type were generated by an initial adhesion step in which 1.2 × 10^6 cells were plated per 75-cm² culture flask in DMEM plus 20% FCS (20 min, 37°C). The nonadherent cells (cardiac myocytes) were centrifuged and then plated at ~1.2 × 10^6 cells per 75-cm² culture flask and grown in 5% CO₂ at 37°C. After 4 days in culture, spontaneous contraction (30–40 beats per min) was observed under phase-contrast microscopy. Greater than 75% of the cells were stained by a murine monoclonal α-smooth muscle actin (sarcomeric) Ab which is specific for α-skeletal muscle actin and α-cardiac muscle actin. Stains Z lines and dots in stress fibers of skeletal and cardiac muscle, but not in nonsarcomeric muscle elements such as connective tissue, epithelium, nerves, or smooth muscle (3).

To obtain cardiac fibroblasts, the primary isolate was plated in flasks (20 min at 37°C). Fibroblasts at passages 3–5 were routinely used in these studies. A fibroblast enrichment in the cell culture was observed (fibroblasts are rapidly proliferating vs myocytes) which was >90%, as assessed using mAb clone IB10 (F-4771; Sigma-Aldrich, St. Louis, MO) which recognizes fibroblasts.

Affinity-purified Abs and serum IgG

Affinity-purified Abs were prepared as described (4). Briefly, affinity-purified Abs against 52 and 60 kDa SSA/Ro proteins were isolated from serum (of a mother whose child has CHB) by affinity column chromatography and the eluted Abs were tested for specificity by ELISA, immunoblot, and immunoprecipitation. Using a Protein A-IgG isolation kit (Pierce, Rockford, IL), human IgG was isolated from three sources: a healthy control, a patient with systemic lupus erythematosus whose serum contains antinuclear Abs but not anti-SSA/Ro-SSB/La Abs; and a mother whose serum contains Abs to all components of the SSA/Ro-SSB/La complex and whose child had cutaneous manifestations of neonatal lupus. Protein concentration of affinity-purified Abs and IgG fractions was assessed by a protein quantitation kit (Pierce). Affinity-purified Abs and normal human IgG were endotoxin-free as assessed by the E-toxate (Limulus amebocyte lysate) assay (Sigma-Aldrich). Samples are routinely processed by application to Detoxi-Gel endotoxin removing gel (Pierce) to remove any contaminating LPS. These columns lower LPS levels to below 1 pg/ml.

Induction of apoptosis

Cardiocytes were treated to induce apoptosis by plating on tissue culture dishes coated with poly (2)-hydroxyethylmethacrylate (polyHEMA) (10) for 18 h at 37°C. Cells were retrieved and apoptosis was assessed by TUNEL staining using a commercial kit as per the recommendation of the manufacturer (no. 1684817; Boehringer Mannheim, Indianapolis, IN).

Preparation of opsonized and nonopsonized apoptotic cardiocytes

Apoptotic fetal cardiocytes were incubated for 30 min with affinity-purified Abs reactive with 52 and 60 kDa SSA/Ro (1 μg/ml) or IgG (5 μg/ml) from the mother whose child had cutaneous neonatal lupus (“opsonized”), or with purified IgG (5 μg/ml) from a healthy control or from a patient with systemic lupus erythematosus absent anti-SSA/Ro-SSB/La Abs (“nonopsonized”).

Isolation and culture of macrophages

Macrophages were derived from PBMCs of healthy donors or from white blood cell concentrate (Leukopak; New York Blood Center, New York, NY) as described (11). Mononuclear cells were isolated by centrifugation on Ficoll-Hypaque gradient. Cells were then suspended in 10 ml of RPMI 1640 + 10% human serum and incubated in Falcon T-75 tissue culture flasks for 1 h at 37°C. Nonadherent cells were removed by washing, leaving an adherent cell population which was retrieved and cultured in Teflon beakers (RPMI 1640 + 10% human serum, 7 days). The purified cell population consisted of >90% monocyte-derived macrophages as measured by phagocytosis of IgG-coated sheep RBC (data not shown).

Macrophages (10⁵/well) were added to polyHEMA plates in the absence or presence of wells containing apoptotic cardiocytes (nonopsonized or opsonized). Supernatants (conditioned media) from the cocultures were collected and analyzed separately. The macrophages from each condition were retrieved, fixed with parafomaldehyde, and stained with the following primary Abs (at 1/200 dilution): mouse IgG (isotype control), ligand-induced binding site 1 (generous gift from M. H. Ginsberg, The Scripps Research Institute, La Jolla, CA), anti-α, β integrin (Chemicon International, Temecula, CA). After addition of anti-mouse IgG FITC, the samples were analyzed by FACS analysis (FACScan) or by indirect immunofluorescence using phase contrast microscopy.

Assessment of fibroblast phenotype and proliferation

To evaluate the phenotype of the cultured fibroblasts, cells were plated on glass coverslips and treated with supernatants generated as described above. Cells were fixed with paraformaldehyde, and permeabilized with 100% acetone. Primary Abs at 1/200 dilution reactive with SMAc (Sigma-Aldrich) were added. After addition of anti-mouse IgG FITC (Sigma-Aldrich), the samples were analyzed by FACS (FACScan) or by indirect immunofluorescence. To evaluate proliferation, cells were assessed via a fluorescent measurement of [3H]thymidine incorporation by liquid scintillation spectrometry.

Statistical analysis

The Student t test for unpaired data was used to compare proliferation and FACS mean fluorescence measurements between the different groups. Values of p < 0.05 were considered significant.
Results

Immunostaining of heart from autopsy study of neonate with CHB

The clinical description and routine postmortem evaluation of a term male infant, diagnosed with AV block at 19 wk and dying at birth, have previously been published (7). Available slides were immunostained with Abs to SMAc (for detection of myofibroblasts) and CD68 (for detection of macrophages). Representative photomicrographs are shown in Fig. 1. As expected, SMAc-positive smooth muscle cells lined the blood vessels. Notably, the ventricular tissue contained numerous areas of fibrosis and microcalcification (Fig. 1, A and B) in which a predominant SMAc-positive infiltrate, indicative of myofibroblasts, was readily observed (Fig. 1, C and D). Small clusters of macrophages could also be appreciated in areas of scar tissue (Fig. 1, G and H). In contrast, there were no SMAc-positive cells (other than those lining the blood vessels) in ventricular tissue from an otherwise healthy 24-wk abortus (Fig. 1E) or a term neonate dying at birth of noncardiac causes (Fig. 1F). The CD68 Ab showed no immunostaining in the subendocardial zones shown (Fig. 1, I and J). Based on these novel findings, in vitro

![Figure 1](image1.png)

**FIGURE 1.** Detection of macrophages and myofibroblasts in CHB. Longitudinal sections through the left ventricle from the affected neonate were stained using H&E (A and B), anti-SMAc (C and D), and anti-CD68 (G and H). Densely packed myofibroblasts (C and D, SMAc-positive cells) are present in thickened fibrous subendocardial areas adjacent to small clusters of macrophages (G and H, CD68-positive cells). Sections from the left ventricle of a normal 24-wk abortus were also stained with anti-SMAc (E) and anti-CD68 (I), as were sections from the right ventricular endomyocardium (one-third of the way from AV valve to apex) of a term neonate dying at birth of noncardiac causes (F, anti-SMAc; J, anti-CD68).

![Figure 2](image2.png)

**FIGURE 2.** Accessibility of SSA/Ro proteins on apoptotic cardiocytes to maternal autoantibodies. Proliferating cardiocytes (plated on collagen type I) or apoptotic cardiocytes (plated on polyHEMA) were incubated with affinity-purified anti-52 and -60 kDa SSA/Ro Abs for 30 min at 37°C, fixed with paraformaldehyde, and stained with Hoechst (DNA) or FITC anti-human IgG. In these nonpermeabilized preparations, SSA/Ro proteins are accessible to maternal autoantibodies in apoptotic (B) but not proliferating cardiocytes (A). Apoptotic cardiocytes were incubated with normal human IgG (C) or with affinity-purified anti-52/60 kDa SSA/Ro Abs (D) for 30 min at 37°C and, after staining with FITC anti-human IgG, were analyzed by FACS. Control cells exhibited uniform symmetrical Hoechst staining and no reactivity to anti-52 and -60 kDa SSA/Ro Abs. In contrast, the polyHEMA treatment resulted in markedly condensed nuclear staining and strongly positive surface staining of SSA/Ro.
experiments were initiated to examine the role of maternal anti-SSA/Ro Abs in the pathologic cascade leading to fibrosis.

Accessibility of SSA/Ro proteins to maternal autoantibodies in proliferating or apoptotic cardiac myocytes

Three different culturing conditions (plates coated with type I collagen, vitronectin, or polyHEMA) were used to address adhesion (crystal violet assay) and serum-induced proliferation ([3H]thymidine incorporation) of the primary fetal cardiocytes. Cardiac myocytes attached to collagen-coated (110% proliferation vs plastic alone) and vitronectin-coated surfaces (127%) but not polyHEMA-coated surfaces (2%). The latter occurred, in part, because serum protein is unable to adsorb to the polyHEMA surface (10). After 48 h, the thymidine incorporation by cardiac myocytes plated on collagen was equivalent to that by myocytes plated on vitronectin (8430 ± 2010 cpm vs 7200 ± 180 cpm; p = NS). In contrast, polyHEMA did not support proliferation of the cardiocytes. This effect reflects a viability requirement involving anchorage signals. At 18 h, >90% of the polyHEMA-plated cardiocytes, which rounded up in aggregates, were TUNEL-positive, supporting that the cells were apoptotic (not shown).

Subsequent experiments confirmed that SSA/Ro proteins were expressed on the surface of fetal cardiac myocytes rendered apoptotic by culture on polyHEMA-coated plates, and were thus accessible to maternal autoantibodies. As assessed by indirect immunofluorescence with affinity-purified Abs, there was no surface staining of normally proliferating cardiocytes (Fig. 2A). In contrast, surface staining was readily demonstrated on polyHEMA-apoptotic cardiocytes incubated with the same affinity-purified Abs (Fig. 2B). As shown by flow cytometry, the polyHEMA-apoptotic cells were not rendered nonspecifically sticky, because there was no staining with an IgG fraction from a normal healthy donor (Fig. 2C), but only with affinity-purified Abs (Fig. 2D).

**FIGURE 3.** Effect of opsonized apoptotic cardiocytes on macrophage activation (FACSscan). Cardiocytes (4 × 10^5/well) were rendered apoptotic by culture on polyHEMA for 18 h at 37°C. Nonopsonized or opsonized apoptotic cardiocytes were prepared by preincubation for 30 min at 37°C with normal human IgG or with affinity-purified anti-52/60 kDa SSA/Ro Abs, respectively. Macrophages (2 × 10^3/well) were incubated with nonopsonized (A and C) or opsonized apoptotic cardiocytes (B and D) for 18 h at 37°C, fixed with paraformaldehyde, stained with LIBS1 (A and B) or αvβ3 (C and D) and FITC anti-mouse IgG (isotype control = IC), and analyzed by FACS. Results are representative of three experiments.

**FIGURE 4.** Effect of supernatants from cocultures of macrophages on transdifferentiation (SMAc staining) of cardiac fibroblasts. Cultured fibroblasts (2 × 10^3/well) were incubated in the absence (A and E) or presence (B–D and F–H) of supernatants diluted 1/1 in DMEM plus 2% FCS (48 h, 37°C). The supernatants were derived from cultured macrophages alone (B and F), macrophages incubated with nonopsonized apoptotic cardiocytes (C and G), or macrophages incubated with opsonized apoptotic cardiocytes (D and H). Fibroblasts were then fixed with paraformaldehyde, stained with Hoechst and anti-SMAc-Cy3, and analyzed by fluorescence microscopy. Results are representative of five experiments. Magnification is ×20 (A–D) and ×40 (E–H).

Activation state of macrophages cocultured with opsonized apoptotic cardiocytes

The next set of experiments addressed whether the opsonized apoptotic cardiocytes would induce macrophage activation. Macrophages were isolated from PBMC of several healthy donors and cocultured for 18 h with opsonized polyHEMA-apoptotic cardiocytes (incubated with affinity-purified Abs to 52 and 60 kDa SSA/Ro) or with nonopsonized polyHEMA-apoptotic cardiocytes (incubated with normal serum IgG). Supernatants (conditioned media) from the cocultures were collected and analyzed separately for viability and activation (FACScan). Cardiocytes (4 × 10^5/well) were rendered apoptotic by culture on polyHEMA for 18 h at 37°C. The next set of experiments addressed whether the opsonized apoptotic cardiocytes would induce macrophage activation. Macrophages were isolated from PBMC of several healthy donors and cocultured for 18 h with opsonized polyHEMA-apoptotic cardiocytes (incubated with affinity-purified Abs to 52 and 60 kDa SSA/Ro) or with nonopsonized polyHEMA-apoptotic cardiocytes (incubated with normal serum IgG). Supernatants (conditioned media) from the cocultures were collected and analyzed separately for viability and activation (FACScan). Cardiocytes (4 × 10^5/well) were rendered apoptotic by culture on polyHEMA for 18 h at 37°C.
for their effects on cardiac fibroblasts (see below). The macrophages from each condition were fixed in 4% paraformaldehyde and evaluated for surface expression of the activation epitope of αβ1 using the mAb LIBS1.

Co-culture of macrophages for 18 h with opsonized apoptotic cardiocytes resulted in a three-fold increase in the expression of active αβ1 over that observed on the macrophages alone (p < 0.01, Fig. 3). In contrast, expression of total αβ1 was similar between macrophages incubated with nonopsonized apoptotic cardiocytes and macrophages incubated with opsonized apoptotic cardiocytes (p = NS).

Effect of supernatant from various culture conditions on fibroblast phenotype

Subsequent experiments addressed whether fibrosis might be a consequence of macrophage activation following phagocytosis of opsonized apoptotic cardiocytes. Primary cultures of fibroblasts were incubated in the presence or absence of supernatants from cocultures of apoptotic cardiocytes and macrophages, fixed in 4% paraformaldehyde, stained using anti-SMAC or monoclonal anti-human fibroblast surface protein (clone 1B10), and analyzed by indirect immunofluorescence. Under basal conditions the cultured fibroblasts did not express SMAC (Fig. 4, A and E). Fibroblasts incubated with supernatants from isolated macrophage cultures alone marginally expressed SMAC (Fig. 4, B and F), which was completely abrogated with medium from cocultures of macrophages and nonopsonized apoptotic cardiocytes (Fig. 4, C and G). In contrast, fibroblasts incubated with supernatant derived from macrophages plus opsonized apoptotic cells expressed markedly increased SMAC (Fig. 4, D and H). In all four conditions (basal, supernatant from macrophages alone, supernatant from macrophages cocultured with nonopsonized apoptotic cardiocytes, and supernatant from macrophages cocultured with opsonized apoptotic cardiocytes), total reactivity by anti-fibroblast surface protein expression was equivalent (not shown). Additional nonopsonized and opsonized conditions were used. The former included an IgG fraction isolated from the serum of a patient with systemic lupus erythematosus who had antinuclear Abs but no anti-SSA/ Ro-SSB/La Abs. Supernatants generated from macrophages cocultured with these apoptotic cardiocytes were added to the fibroblasts and no SMAC staining was observed (data not shown). The latter included an IgG fraction isolated from the serum of a mother with Abs to all components of the SSA/Ro-SSB/La complex and whose child had cutaneous manifestations of neonatal lupus. Equivalent results were obtained with affinity-purified anti-52/60 Ssa/Ro Abs, cocultures with these apoptotic cardiocytes markedly increased SMAC staining of the fibroblasts (data not shown).

To examine the potential role of TNF-α and TGFβ in the transdifferentiation of cardiac fibroblasts by the opsonized supernatant, the effects of these two cytokines and their neutralizing Abs were evaluated. The direct addition of TNF-α to the fibroblast cultures did not result in SMAC expression (Fig. 5A). In contrast, addition of TGFβ alone markedly increased expression of SMAC (Fig. 5B). As might be predicted by these results, TNF-α-neutralizing Ab had no effect when added to the macrophage opsonized supernatant (Fig. 5D). However, incubation of the same macrophage supernatant with a neutralizing anti-TGFβ Ab resulted in complete attenuation of the elicited SMAC expression (Fig. 5E).

Proliferation was used as an additional readout of fibroblast phenotype, because the proliferative phase is critical to the formation of granulation tissue during wound healing. The basal proliferation of cultured fibroblasts was decreased in cells cocultured with conditioned medium derived from macrophages plus nonopsonized apoptotic cells (10,346 vs 7,139; p = 0.012). In contrast, when fibroblasts were cocultured with conditioned medium obtained from macrophages plus opsonized apoptotic cells, proliferation was significantly enhanced (12,320 cpm ± 2,008 SE (opsonized) vs 7,139 cpm ± 590 SE (nonopsonized); p < 0.05, n = 9).

To examine the potential role of TNF-α and TGFβ in the stimulated proliferation of cardiac fibroblasts by supernatants, both cytokines and their respective blocking Abs were added as was done for evaluation of SMAC expression. TNF-α did not affect basal proliferation.
fibroblast proliferation, paralleling the results obtained for expression of SMAc. However, contrary to its effect on SMAc, TGFβ significantly inhibited proliferation (4,378 cpm ± 360 SE vs 1,857 cpm ± 297 SE; p < 0.001) (Fig. 6). Correspondingly, TGFβ-neutralizing Ab reversed this effect and further increased the proliferative response observed when fibroblasts were exposed to the macrophage-opsonized supernatant.

**Discussion**

A molecular scenario in which maternal anti-SSA/Ro-SSB/La Abs convincingly contribute to the pathogenesis of cardiac scarring has yet to be formulated. Two points, one clinical and the other cellular, are particularly difficult to reconcile. Only 2% of neonates born to mothers with the candidate Abs have CHB (13), yet these Abs are present in over 85% of mothers whose fetuses are identified with conduction abnormalities in a structurally normal heart (1). Accordingly, the Abs are necessary but insufficient to cause CHB. The final pathway leading to fibrosis may be variable, kept totally in check in most fetuses (normal sinus rhythm), subclinical in others (first degree block) and fully executed in very few (advanced block). The intracellular location of the target Ags raises questions regarding accessibility to maternal Abs. In this study, the capacity of maternal Abs to initiate an inflammatory cellular response in the target tissue and promote fibrosis was addressed, and the following demonstrated: 1) cardiac myocytes rendered apoptotic by culturing on polyHEMA express 52 and 60 kDa SSA/Ro on the surface and can be opsonized by the cognate maternal Abs; 2) macrophages incubated with these opsonized apoptotic cardiocytes become activated; and 3) macrophage-derived factors induce phenotypic changes in cardiac fibroblasts supportive of scarring.

Previous studies to define the pathogenesis of Ab-mediated insult have focused on apoptosis, which may account for the problem of accessibility in the fetal heart and the absence of injury in the maternal heart. Indeed, Tran et al. (14) have recently identified physiologic apoptosis and translocation of SSB/La in the developing murine heart. In this study, we demonstrate that cardiac myocytes readily undergo apoptosis when plated on polyHEMA, which reflects their enhanced sensitivity to apoptotic stimuli. The apoptotic myocytes expressed SSA/Ro and SSB/La on the surface and were opsonized by the cognate maternal Abs. This confirms earlier studies in which staurosporine and 2,3-dimethoxy-1,4-naphthoquinone resulted in apoptosis of cardiac myocytes and binding by anti-SSA/Ro-SSB/La Abs (3, 4). However, it is readily acknowledged that the specificity of these particular Abs for cardiac damage remains to be accounted for. Perhaps other autoantigens (such as Sm or lamin B) which do translocate to the apoptotic blebs (15, 16) are not truly accessible on the surface.

Inadvertent opsonization of the apoptotic cardiac myocyte could pathologically modify the scavenging macrophages with regard to their activation state, signaling pathways and release of soluble mediators. Precedent for early involvement of macrophages is supported by the autopsy findings of a 19-wk fetus with CHB. Histologic studies disclosed dense lymphohistiocytic infiltrates and myocyte degeneration in the cardiac conduction system including the AV node and bundle of His (7). In the present study, coincubation with opsonized apoptotic cardiocytes resulted in a phenotypic change of the macrophage as evidenced by increased expression of an activation epitope of α,β, Relative to the influence on signaling pathways, phosphorylation and nuclear translocation of p44/p42 MAP kinase (extracellular-regulated kinase has recently been demonstrated in extracts of macrophages cocultured with apoptotic cardiocytes preincubated with affinity-purified Abs or IgG fractions reactive with 48 kDa SSB/La, and 52 and 60 kDa SSA/Ro, but not in macrophages cocultured with apoptotic cardiocytes preincubated with normal human IgG.4

The effect of macrophage activation on the cardiac fibroblast may be the critical component leading to fibrosis of the AV node (and in some cases extending to other components of the conduction system and even the working myocardium). Cross-talk between macrophages and fibroblasts, and consequent tissue fibrosis, has been emphasized in other organs. In experimental unilateral ureteral obstruction, the clearance of apoptotic tubular cells by macrophages resulted in the appearance of proliferating SMAC-positive myofibroblasts (17). In models of canine hepatic and renal injury, macrophages are present in less extensive areas of fibrosis, and myofibroblasts dominate in the more advanced grades of fibrosis (18). In a murine model of granulomatous experimental autoimmune thyroiditis, fibrosis was considered secondary to macrophages which produced TGFβ1 (19). Interestingly, however, the irreversible fibrotic replacement of normal conducting tissue may be unique to autoantibody-associated CHB. Other inflammatory stimuli, e.g., those that occur with Lyme disease; generally induce transient AV nodal block (20, 21). Although this may reflect a more rapid resolution of the inflammatory process than occurs with

CHB, it suggests that fibroblast transdifferentiation is not merely a final common pathway of inflammation. Dedifferentiation of fibroblasts is associated with scar formation. Fibrosis is due to a persistent myofibroblast, a phenotype associated with wounding. The transient appearance of a myofibroblast is recognized as a pivotal component of granulation tissue formation (6). Proliferation of myofibroblasts is accompanied by the expression of SMαC and the embryonal isofrom of smooth muscle myosin H chain, and by release of types I and III collagen. Thus, the myofibroblast functionally resembles features of both smooth muscle cells and fibroblasts (22). A recent study compared the phenotype of myofibroblasts and normal fibroblasts from a patient with a recurrent abdominal incision wound herniation (23); myofibroblasts were distinguished from fibroblasts by their increased expression of SMαC and of α5β1 integrin. This phenotype may contribute to unresolved scarring because the binding of collagen to α5β1 integrin interferes with lattice contraction and also prevents the organization of matrix (an α5 integrin-collagen interaction). Unresolved scarring occurs in the heart following diverse modes of injury, e.g., pressure-overloaded left ventricle hypertrophy and a mouse model of autoimmune myocarditis (immunized with cardiac myosin) (24).

The precise composition of the supernatants (inclusive of cytokines, superoxide radicals, etc.) obtained from macrophages cocultured with opsonized cardiocytes has yet to be determined. Based on our previously reported finding of TNF-α release (4), and the association of TGFβ with clearance of apoptotic cells (25, 26), initial experiments evaluated these two cytokines and their neutralizing Abs. The present study is the first to demonstrate increased SMαC expression in human fetal cardiac fibroblasts after incubation with TGFβ, and is supported by a previous report in which s.c. injection of rats with TGFβ, but not TNF-α, resulted in the formation of granulation tissue with an abundance of SMαC-expressing cells (27). Although our data support a role for TGFβ in the transdifferentiation to a myofibroblast, this cytokine clearly inhibited proliferation. In contrast, TNF-α did not induce any measurable changes in the cultured fibroblasts. Whether activation of the cytokines or their respective receptors on the fibroblasts is relevant remains to be evaluated. Moreover, the ratio of various cytokines in the opsonized supernatants may be another determining factor. In contrast, macrophages cocultured with nonopsonized apoptotic cells secrete factors that do not induce a myofibroblast phenotype and actually down-regulate proliferation. Importantly, this latter scenario likely represents the physiologic process of remodeling of the human heart during fetal development, in which apoptotic cells are rapidly cleared with no inflammatory sequelae.

Histopathologic studies support that extensive fibrosis occurs in autoantibody-associated CHB. For example, the spectrum of CHB in the clinically affected fetus includes AV nodal replacement by fibrosis or fatty tissue (5), fibrous structures containing microscopic crystalline structures in the conduction system (28), and altered contractility of the working myocardium secondary to endocardial fibroelastosis (29). The immunostaining of the heart described in this study is the first to demonstrate the presence of myofibroblasts in the region of scarring. The selective vulnerability of the heart (in addition to the skin and less commonly the liver) may relate to the susceptibility of the cardiac fibroblast to transdifferentiation. Of interest, pulmonary abnormalities have not been described as part of the spectrum of neonatal lupus. In this study, although the transdifferentiation of cardiac fibroblasts was readily demonstrated, the same supernatants were incapable of transdifferentiating fetal lung fibroblasts and did not cause their proliferation (R. M. Clancy, A. D. Askasane, R. P. Kapur, E. Chiopelas, N. Azar, M. E. Miranda-Carus, and J. P. Buyon, unpublished observation).

The studies described represent a novel view of the cardiac fibroblast as a fetal factor in autoantibody-associated CHB. Taken together, these in vivo and in vitro data support the speculation that CHB results from unresolved wound healing subsequent to the transdifferentiation of cardiac fibroblasts into proliferating myofibroblasts, a pathologic process initiated by specific maternal Abs. AV nodal cells and even the working myocardium may be particularly vulnerable to the myofibroblast, ultimately leading to fibrosis. It is acknowledged that this in vitro study may be an exaggerated model only applicable to the more severely affected cases, such as the neonate described in this study whose tissue was available for immunohistology. Thus, a reasonable prediction is that there are both susceptibility and regulatory factors, such as fetal polymorphisms of FcR and cytokines, each of which could influence the extent of the proposed pathologic cascade to result in permanent third degree heart block. Dissecting the individual components should provide insights into the pathogenesis of Ab-associated CHB and the rarity of irreversible injury.

References


