

## Immunohistologic Evidence Supports Apoptosis, IgG Deposition, and Novel Macrophage/Fibroblast Crosstalk in the Pathologic Cascade Leading to Congenital Heart Block

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**Objective.** To assess *in vivo* the pathologic cascade leading to fibrosis in congenital heart block (CHB). *In vitro* studies suggest that CHB is initiated via apoptosis, resulting in translocation of SSA/Ro and SSB/La antigens and surface binding by maternal autoantibodies. These opsonized cardiocytes are phagocytosed by macrophages, which secrete factors inducing fibrosis.

**Methods.** Immunohistochemistry analysis was performed on formalin-fixed sections of 4 fetal hearts identified *in utero* as having CHB or isolated myocarditis; mothers had anti-SSA/Ro and anti-SSB/La antibodies.

**Results.** Apoptosis was most extensive in fetuses dying early and most pronounced in regions containing conduction tissue. Deposition of IgG was observed in hearts from fetuses with CHB/myocarditis, but not in 3 control hearts, and was colocalized with apoptotic cells. Giant cells and macrophages (frequently seen proximal to IgG and apoptotic cells) were present in septal and thickened fibrous subendocardial regions, most apparent in the youngest fetuses. Septal tissue also revealed extensive areas of fibrosis and microcalcification in which a predominant smooth muscle actin (SMA)-positive infiltrate (myofibroblast scarring phenotype) was observed. In contrast, there were no macrophages or SMA-positive cells (other than those lining blood vessels) in septal tissue from control hearts, although

rare macrophages were seen in the working myocardium.

**Conclusion.** In summary, findings in this unique autopsy material paralleled those in *in vitro* studies. These data support the notion of exaggerated apoptosis, probably due to ongoing inflammation caused by IgG binding and ingestion by macrophages. Transdifferentiation of cardiac fibroblasts to a scarring phenotype may be a pathologic process initiated by maternal antibodies, and persistence of this phenotype even after birth may relate to the progression of block seen in some infants postpartum.

The identification of congenital heart block (CHB) in a fetus, particularly in the late second trimester and in the absence of structural abnormalities, predicts with at least 85% certainty that the mother will have autoantibodies to components of the intracellular SSA/Ro–SSB/La system (1). With increasing awareness of this association, it has become evident that the spectrum of cardiac injury not only includes first- to third-degree block, but can also extend to the myocardium and endocardium (2). The pathologic cascade to irreversible fibrosis, characteristic of autoimmune-associated CHB, has been difficult to define at the molecular level. The challenge rests on integrating the initial antibody insult with the final cardiac injury and reconciling the fact that most infants born to mothers with the candidate antibodies do not have clinically detectable atrioventricular (AV) block (3). The pathway to scarring may be variable: kept totally in check in most fetuses (normal sinus rhythm), remaining subclinical in others (first-degree block), and becoming fully executed in a very few (advanced block). Moreover, CHB is an injury unique to some phase(s) of development, since it has never been reported in the maternal heart despite the presence of identical antibodies in the maternal circulation.

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In vitro studies employing cardiac myocytes and fibroblasts separately isolated and cultured from human fetal hearts provide evidence for a pathologic link between antibodies and injury (4). After induction of apoptosis, SSA/Ro and SSB/La translocate from their normal intracellular residence to the surface of apoptotic blebs, where they can be bound by their cognate antibodies and become opsonized (5,6). Cocultured macrophages have been shown to phagocytose opsonized apoptotic cardiocytes and to secrete inflammatory cytokines such as tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) (6). Other investigators have also demonstrated that phagocytosis of opsonized apoptotic cells is proinflammatory (7,8), as exemplified by the observation that ingestion of apoptotic cells bound by anticardiolipin antibodies results in the release of TNF $\alpha$  from cocultured macrophages (8). Human fetal cardiac fibroblasts exposed to supernatants obtained from macrophages incubated with opsonized apoptotic cardiocytes markedly increased expression of the transdifferentiation myofibroblast marker smooth muscle actin (SMA), associated with scarring, an effect blocked by neutralizing anti-transforming growth factor  $\beta$  (anti-TGF $\beta$ ) antibodies (4).

Validation of the in vitro model described above has been limited by the availability of histologic specimens from hearts of fetuses dying of CHB, since the mortality rate is relatively low (2) and death most often occurs weeks to months after initial detection of bradyarrhythmia. However, immunohistologic study of the heart of a full-term male infant first diagnosed as having AV block at age 19 weeks supported the notion of macrophage crosstalk, despite a 5-month interval from detection to death (4). Ventricular tissue revealed microcalcification in which a predominant SMA-positive infiltrate was seen. Macrophages were also observed in areas of scar tissue. Notably, the fibrosis was not bland, but involved an infiltrate of activated myofibroblasts months after the initial insult. To confirm and extend these findings, this and 3 other hearts, encompassing a spectrum of disease severity and timing of death relative to clinical detection, were extensively examined. The present study represents a first-time evaluation of each component of the proposed pathologic cascade to scarring. Accordingly, the focus was on the extent of apoptosis, IgG deposition, macrophage accumulation, and fibroblast phenotype.

## PATIENTS AND METHODS

**Specimens.** These studies were approved by the Institutional Board of Research Associates. Affected and control

hearts were obtained as follows (clinical characteristics of affected specimens are also summarized in Table 1).

*22-week-old fetus with CHB.* This female fetus was diagnosed at fetal age 22 weeks as having third-degree block and the pregnancy was electively terminated. The gross anatomy of the heart was normal. The valves, septa, and outflow tracts appeared grossly normal. The mother was a 27-year-old white woman (G1P0) who was diagnosed as having lymphopenia on routine examination 2 years prior to the pregnancy in question. She was asymptomatic at that time and also found to have positive antinuclear antibodies (1:160) and anti-SSA/Ro and anti-SSB/La antibodies. At the time of the pregnancy, the complement levels were normal, and tests for anti-double-stranded DNA (anti-dsDNA), anti-Sm, anti-RNP, and anticardiolipin antibodies yielded negative results. She received no treatment with dexamethasone prior to the termination of the pregnancy.

*20-week-old fetus with CHB.* This female fetus was diagnosed at fetal age 18 weeks as having third-degree block and hydrops and died within 2 weeks despite receiving maternal oral dexamethasone at 4 mg/day for several days. The gross anatomy was normal. Microscopic sections of the heart showed autolysis with fragmentation of the myocardium and loss of nuclear staining. Despite the autolytic changes, the overall architecture of the organ was preserved. The mother was a 36-year-old white woman (G3P1) who had previously had a miscarriage at 7 weeks. She was diagnosed as having Sjögren's syndrome and was found to have antibodies to SSA/Ro and SSB/La 18 months after her first pregnancy.

*34-week-old fetus with myocarditis.* This female fetus died suddenly at fetal age 34 weeks. There was no evidence of heart block. The autopsy unexpectedly revealed pancarditis (without infection). The gross anatomy of the heart was normal. The mother was a 38-year-old Asian woman (G6P1) and had an undifferentiated autoimmune syndrome consisting of dry eyes (no objective testing done) and arthralgias. Serologic examination of the mother revealed antibodies to SSA/Ro and SSB/La, possible rheumatoid factor, low-positive IgG, anticardiolipin antibodies, no anti-dsDNA antibodies, and normal complement levels.

*Neonate with CHB (40-week gestation).* This previously reported (4,9) male neonate, diagnosed as having an enlarged right ventricle (RV) at fetal age 19 weeks and third-degree block at 24 weeks, died at 40 weeks (2 hours postdelivery). The mother was a 30-year-old white woman (G1P0) with antibodies to SSA/Ro and SSB/La; her rheumatologic health status was unknown. The neonate's heart was irregularly shaped with a hypoplastic RV, dilated left ventricle (LV), and enlarged right atrial appendage. The right atrial wall was thickened, and an atrial septal defect (secundum type) existed. The anterior surface of the RV, adjacent to the interventricular septum, was indented and contained multiple aneurysmal outpouchings. The RV was moderately hypoplastic with diffuse endocardial fibroelastosis, and the interventricular septum grossly contained foci of fibrosis. The LV was slightly dilated. The tricuspid and pulmonic valves contained numerous nodules along their cusps (polyvalvular dysplasia). The mitral and aortic valves were grossly normal. There were clear pleural and pericardial effusions.

**Table 1.** Clinical description of 4 fetuses diagnosed as having congenital heart block or myocarditis in utero\*

Time of detection (diagnosis)	Time of death (cause)	Treatment	Maternal antibodies	Sex
22 weeks (3° block)	22 weeks (elective termination)	None	Anti-La 48 kd, anti-Ro 52/60 kd†	F
18 weeks (3° block, hydrops)	20 weeks (demise)	Dexamethasone	Anti-La 48 kd, anti-Ro 52 kd†	F
34 weeks (myocarditis)	34 weeks (demise)	None	Anti-La 48 kd, anti-Ro 52/60 kd†	F
19 weeks (enlarged RV), 24 weeks (3° block)	40 weeks (demise 2 hours postdelivery)	Dexamethasone	Anti-Ro/La‡	M

\* 3° = third-degree; RV = right ventricle.

† Determined in clinical laboratory at the Hospital for Joint Diseases using a Diamedix kit; additionally, a sodium dodecyl sulfate blot of Molt-4 cells was performed as described previously (27).

‡ Determined at an unspecified commercial laboratory.

**Controls.** Hearts were obtained, following elective termination of pregnancy at 22, 23, and 24 weeks of gestation, from 3 fetuses in which there was no known cardiac disease. Also evaluated were available cardiac sections from a full-term (40 weeks of gestation) newborn, previously reported (4), who died of noncardiac causes shortly after birth.

**Preparation of slides.** Formalin-fixed, 6- $\mu$ m paraffin sections were obtained from all hearts. Sections of RV, LV, and AV groove/conduction (septal) tissue were obtained from the 20-week CHB, 22-week CHB, and 22-week control hearts. Right and left ventricular sections were obtained from the 34-week myocarditic, 40-week CHB neonatal, 23-week control, 24-week control, and 40-week control hearts. Deparaffinization was achieved by warming up paraffin sections (60°C, 1 hour). Sections were then sequentially treated with xylene and ethanol (30 minutes each, 22°C).

**Immunostaining.** Endogenous peroxidase was inactivated by treatment with methanol + 1% H<sub>2</sub>O<sub>2</sub> (30 minutes, 22°C). Sections were washed with phosphate buffered saline (PBS; 15 minutes, 2 $\times$ ). Demasking of epitopes was achieved by digestion with 1 mg/ml Type IV-S hyaluronidase (H-3884; Sigma, St. Louis, MO) in 0.1M Tris, pH 6.0, for 30 minutes at 22°C. Slides were once again incubated with PBS (5 minutes, 3 $\times$ ), and nonspecific sites were saturated by treatment with 5% normal goat serum in PBS (30 minutes, 22°C). Apoptosis was assessed using the In Situ Cell Death Detection Kit (1 684 817; Boehringer-Mannheim, Mannheim, Germany). This assay utilizes the TUNEL technique. Fluorescein isothiocyanate (FITC) incorporation was assessed by fluorescence microscopy. Alternatively, the dye method employed peroxidase-conjugated anti-FITC and utilized a counterstain with light red (Congo red, 50-130; Rowley Biochemical, Danvers, MA). The apoptotic index (AI), a quantitative measure of apoptosis, was expressed as AI = (TUNEL-positive nuclei/total nuclei)  $\times$  100, where the total number of nuclei is the number of nonapoptotic nuclei (purple) plus the number of apoptotic (TUNEL-positive) nuclei (brown).

Alternatively, slides were incubated overnight at 4°C with primary antibody. Sections were immunostained with alkaline phosphatase (AP)-conjugated anti-human IgG (A3312; Sigma) or with the primary monoclonal antibodies anti-SMA (reacts with smooth muscle cells lining blood vessels and myofibroblasts) (08-1106; Zymed, South San Francisco, CA), anti-CD68 (2165; Immunotech, Marseilles, France), or anti-CD8 (08-1289; Zymed), and then incubated with

peroxidase-conjugated anti-mouse IgG or AP-conjugated anti-mouse IgG (1 hour, room temperature). Slides were visualized using Fast 3,3'-diaminobenzidine tablet sets (D-4168; Sigma) or Fast Red TR/Naphthol AS-JMX tablet sets (F-4648; Sigma) to report peroxidase and AP, respectively. The sections were counterstained with hematoxylin prior to photography.

**Staining for fibrosis.** The picrosirius red staining method was chosen. This approach accurately reflects organ collagen content assessed with hydroxyproline assays and allows areas of localized collagen accumulation to be specifically evaluated (10).

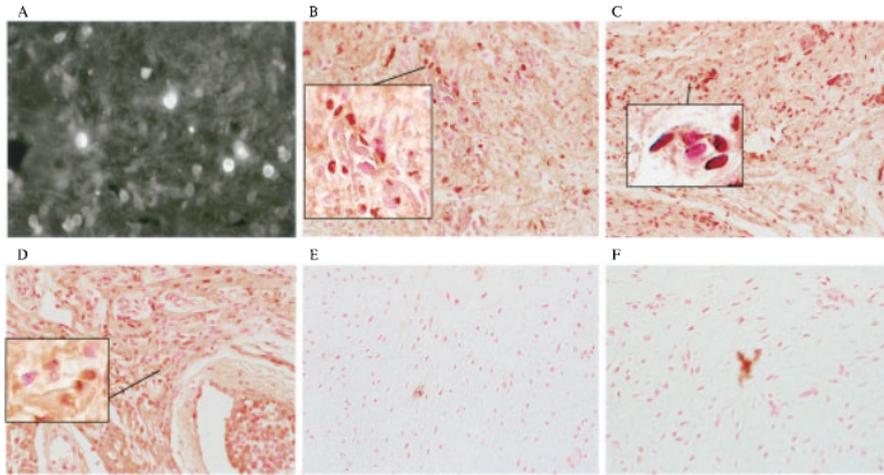
**RESULTS**

**Evaluation of apoptosis and IgG in histologic sections of fetal/neonatal hearts.** As assessed by TUNEL (FITC and immunoperoxidase detection), apoptosis was increased in available sections (including septal tissue, RV, and LV) from hearts of the 20-, 22-, and 34-week-old fetuses with CHB/myocarditis compared with sections from the heart of the neonate with CHB who died at birth and from 22-week and 23-week control hearts (Table 2 and Figure 1). Notably, apoptotic cardiocytes

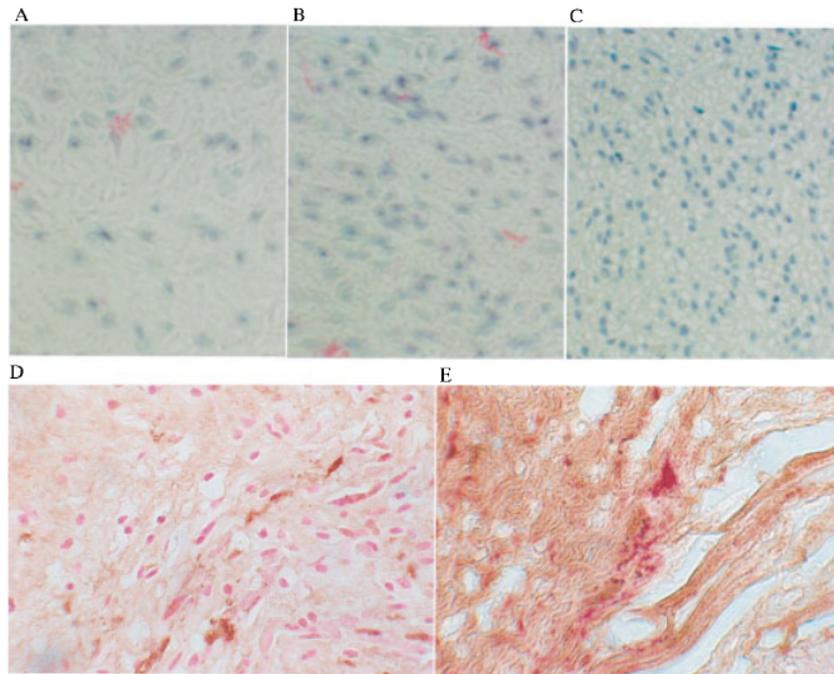
**Table 2.** Evaluation of apoptosis in histologic sections of fetal/neonatal hearts\*

Source of fetal/neonatal heart	Apoptotic index		
	RV	Septum	LV
22-week-old fetus with CHB	9.9 $\pm$ 3.2	37.0 $\pm$ 2.1	7.0 $\pm$ 2.0
20-week-old fetus with CHB	5.2 $\pm$ 1.0	30.5 $\pm$ 3.1	2.8 $\pm$ 2.0
34-week-old fetus with myocarditis	ND	ND	3.0 $\pm$ 1.7
Neonate with CHB who died at birth	ND	ND	0.2 $\pm$ 0.2
Normal 23-week-old fetus	0.3 $\pm$ 0.5	0.2 $\pm$ 0.5	0.3 $\pm$ 0.6

\* Values are the mean  $\pm$  SEM apoptotic index (AI), expressed as AI = (TUNEL-positive nuclei/total nuclei)  $\times$  100, where the total number of nuclei is the number of nonapoptotic nuclei plus the number of apoptotic (TUNEL-positive) nuclei. One hundred cells were counted in 3-5 fields for each cardiac section. RV = right ventricle; LV = left ventricle; CHB = congenital heart block; ND = not determined (since cardiac sections were unavailable).



**Figure 1.** Histologic evidence of increased apoptosis in conduction tissue and ventricle from 2 fetuses with congenital heart block (CHB) and 1 with myocarditis. Shown are longitudinal sections through septum of a 22-week-old fetus with CHB (A and B), septal tissue of a 20-week-old fetus with CHB (C), ventricular tissue from a 34-week-old fetus with myocarditis (D), ventricular tissue from a full-term neonate with CHB who died at birth (E), and ventricular tissue from a normal 22-week-old fetus that was electively terminated (F). Apoptotic cells were identified by TUNEL fluorescein isothiocyanate staining (A) or TUNEL peroxidase (B–F). Apoptotic cells (brown nuclei) are interspersed with nonapoptotic cells (purple nuclei) (insets in B–D). (Original magnification  $\times 40$  in A–F; insets in B–D are photographic enlargements.)



**Figure 2.** Histologic evidence of increased human IgG in conduction tissue and colocalization of human IgG with apoptotic cells in congenital heart block (CHB). Shown are longitudinal sections through septa of 20-week (A) and 22-week (B) CHB hearts stained with alkaline phosphatase-conjugated anti-human IgG. Clusters of human IgG (red staining) surround healthy cells. No IgG was seen in a longitudinal section from a normal 23-week fetal heart (C). The section represented in B was stained with TUNEL peroxidase and counterstained with Congo red (pink = nonapoptotic, brown = apoptotic) (D). The same tissue section represented in B and D was double stained with TUNEL peroxidase (brown) and alkaline phosphatase conjugated to anti-human IgG (red) to demonstrate colocalization of apoptosis and IgG, respectively, and was not counterstained (E). (Original magnification  $\times 40$ .)

were not present in contiguous tracts, but were diffusely scattered between nonapoptotic cells (Figure 1, insets). In the 22-week CHB heart, the AI in septal tissue was 37%, compared with 9.9% for RV and 7% for LV (Table 2). In contrast, tissue from all anatomic regions of the 23-week control heart revealed only scant TUNEL-positive cells consistent with physiologic apoptosis (AI <1% for septum, RV, and LV) (Table 2).

Although IgG deposition was more limited in distribution than apoptosis, it too was greater in the 20-, 22-, and 34-week-old affected fetuses than in the CHB neonate who died at birth. Specifically, IgG staining of the 20- and 22-week CHB fetal hearts was evident in areas proximal to the AV groove (Figures 2A and B) and in the LV of the 34-week-old fetus with myocarditis (not shown). In contrast, IgG was not found in the septum of the normal 23-week fetal heart (Figure 2C) or in the LV of the normal 22-week and 24-week hearts (not shown).

Double staining of various tissue sections revealed that IgG deposition was limited to areas surrounding apoptotic cells. This was particularly evident in septal tissue from the 22-week-old fetus with CHB (Figure 2E).

#### **Evaluation of inflammatory and fibrosing components in histologic sections of fetal/neonatal hearts.**

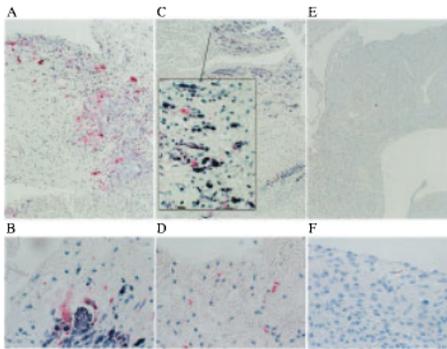
To evaluate an inflammatory component in the pathogenesis of CHB, cardiac sections were assessed for the presence of macrophages. These cells are likely to be involved in the clearance of nonopsonized and opsonized apoptotic cells, presentation of antigen to lymphocytes, promulgation of calcification, and interaction with fibroblasts that induces a scarring phenotype. Macrophages were detected in the hearts of all affected fetuses, most prominently in the early cases. In the 20- and 22-week CHB hearts, CD68-positive staining was present in the septal region (Figures 3A–D). Specifically, small clusters of macrophages were readily observed in areas of fibrosis, which colocalized with IgG deposition (Figure 4A). The next experiments focused on colocalization of macrophages and opsonized apoptotic cardiocytes. Sections from the septal region of the heart obtained from the 22-week-old electively terminated fetus with CHB were double stained for TUNEL and CD68. Macrophages were readily evident and were found to be engulfing opsonized apoptotic nuclear material (Figures 4C–E).

Further support for the notion of an inflammatory component was provided by the detection of macrophage infiltrates characterized by the formation of CD68-positive multinucleated giant cells (Figure 3B) and CD8-positive T lymphocytes (not shown), the latter

appearing only in the 20-week CHB heart and the 34-week myocarditic heart. Moreover, these infiltrates were apparent not only in areas of calcification within the conduction tissue, but also in fibrous subendocardial regions. In the 20-week and 22-week CHB hearts, extensive calcifications with associated chronic inflammation were present in most of the atrial wall, the AV groove, and portions of the epi- and endocardium of the ventricles. These areas comprised closely packed mineralized deposits surrounded by macrophages and multinucleated giant cells (Figures 3A–C). Large macrophages ( $185/\text{cm}^3$ ) and giant cells ( $14/\text{cm}^3$ ) were present in the septum of the 20-week CHB heart. In the 22-week CHB heart, inflammatory infiltrates were also primarily concentrated around calcified deposits, and large macrophages ( $80/\text{cm}^3$ ) and giant cells ( $22/\text{cm}^3$ ) were present in the septum. In contrast, while macrophages were present in the working myocardium of the control 23-week fetal heart, they were smaller in size, rare, evenly distributed, and not anchored to a pathologic process such as microcalcification or IgG deposition (Figure 3F).

For the neonate with CHB who died months after initial detection of bradycardia in utero, available ventricular tissue revealed prominent calcification and fibrosis, but infiltration of macrophages and giant cells was scant (not shown). The localization of these cellular infiltrates was shifted away from the conduction system and was more prominent in the subendocardium. Macrophages were not observed in any tissue sections from the 40-week control heart (from the neonate who died of noncardiac causes). In the 34-week myocarditic heart, macrophages infiltrated the myocardium and, more severely, the endocardium and pericardium (not shown).

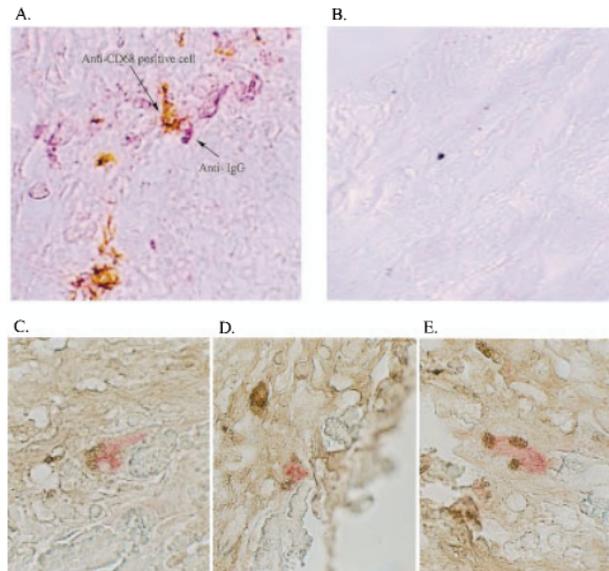
To evaluate the fibrosing component of the pathway leading to cardiac damage, cardiac sections were assessed for the presence of myofibroblasts, transdifferentiated fibroblasts that promote scarring. Myofibroblasts were detected in hearts of all affected fetuses regardless of the timing of death relative to detection (Figures 5A, B, and D). As expected, myofibroblasts were located in areas of fibrosis (Figures 5E and F). Septal sections from the 22-week CHB heart showed myofibroblasts associated with extensive fibrous matrix and marked calcification in the inferior portion of the atrial wall where the AV node is likely to reside (Figures 5A and E). In septal tissue of the 20-week CHB heart, myofibroblasts were also found in the anticipated site of the AV node as well as in thickened fibrous subendocardial areas (Figures 5B, D, and F). Myofibroblasts were observed in the region of scarring in the heart from



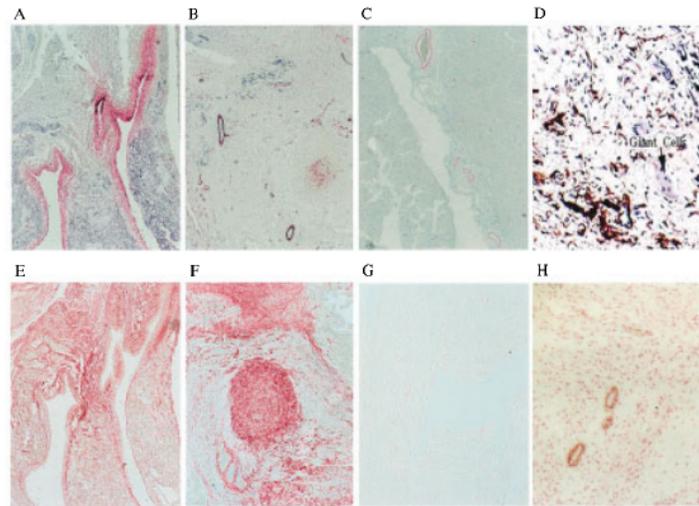
**Figure 3.** Histologic evidence of increased macrophages in conduction tissue of fetal hearts with congenital heart block (CHB). Shown are longitudinal sections through septa of 22-week (A) and 20-week (C) CHB hearts stained with anti-CD68 and alkaline phosphatase-conjugated anti-mouse IgG (red). A 40 $\times$  magnification of A demonstrates multinucleated giant cells surrounding a region of calcification (B). Small clusters of macrophages (red) are demonstrated in areas of scar tissue (inset in C). A 40 $\times$  magnification of C shows an area without calcification (D). A longitudinal section through the septum of a normal 23-week fetal heart (E) and a 40 $\times$  magnification of the same section (F) are also shown. (Original magnification  $\times$  10 in A, C, and E; inset in C is photographic enlargement.)

the neonate with CHB who died at birth (not shown) and were also evident in the LV of the 34-week-old fetus with myocarditis (not shown). There was no evidence of SMA-positive cells (other than those lining blood vessels) or fibrosis in either septal or ventricular tissue from the control fetuses aborted at 22 weeks (not shown) or 23 weeks (Figures 5C, G, and H), or from the full-term neonate who died of noncardiac causes (not shown).

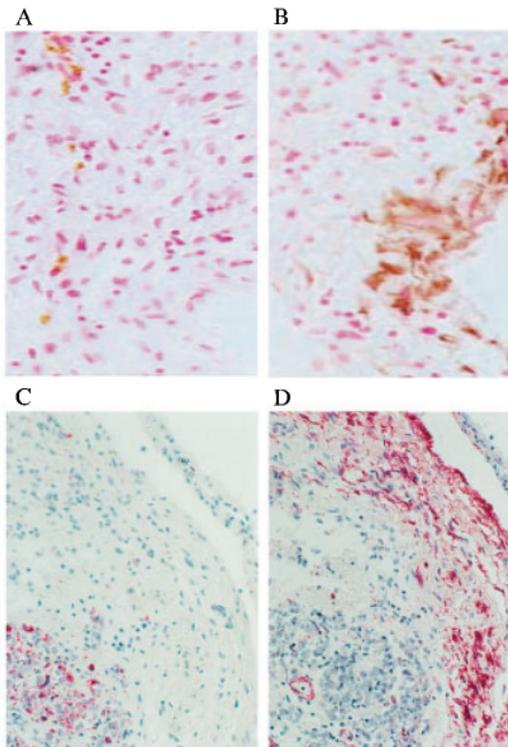
Given data from *in vitro* studies suggesting that factors secreted by phagocytosing macrophages result in the transdifferentiation of the fibroblasts (4), evidence for crosstalk between these cells was sought in the histologic sections. In the 20-week CHB heart, clusters of macrophages in close proximity to myofibroblasts were present in scar tissue near the AV groove as well as the thickened fibrous subendocardium (Figure 6). Interestingly, there was a clear association between macrophages and myofibroblasts based on the colocalization of these cells in all regions of the septal tissue (20- and 22-week CHB hearts, not shown). Although apoptotic cardiocytes localize with macrophages and myofibroblasts, apoptotic cardiocytes were not restricted to areas



**Figure 4.** Colocalization of macrophages and IgG and colocalization of macrophages and apoptotic cells in the septal region of a heart from a 20-week-old fetus with congenital heart block (CHB) and in the septal region of a 22-week CHB heart. Shown are longitudinal sections through the septa of a 20-week CHB heart (A), a 23-week normal heart (B), and a 22-week CHB heart (C–E). A and B, Double staining with anti-CD68 and peroxidase-conjugated anti-mouse IgG (brown) and alkaline phosphatase-conjugated anti-human IgG (red). C–E, Double staining for TUNEL and CD68. Macrophages were found in proximity to human IgG and were engulfing opsonized apoptotic nuclear material. No counterstaining was performed. (Original magnification  $\times$  40.)



**Figure 5.** Histologic evidence of myofibroblasts in conduction tissue of the heart from a fetus with congenital heart block (CHB). Shown are low-magnification images of longitudinal sections through the septa of a 22-week CHB heart (A and E), a 20-week CHB heart (B and F), and a 23-week normal heart (C and G) stained with anti-smooth muscle actin (SMA) and alkaline phosphatase-conjugated anti-mouse IgG (red) (A–C) or picrosirius red (E–G). A 40× magnification of the tissue in B shows staining of myofibroblasts with peroxidase-conjugated anti-mouse IgG (brown) (D). A multinucleated giant cell is seen adjacent to an area of myofibroblasts (D). A 40× magnification of the tissue in C is shown with Congo red counterstaining (H). SMA-positive cells line the blood vessels of both CHB (A and B) and normal (C and H) hearts. E–G, No counterstaining. (Original magnification × 10 in A–C and E–G.)



**Figure 6.** Proximity of macrophages and myofibroblasts in the septa of 2 hearts with CHB. Longitudinal sections through septa of 22-week (A and B) and 20-week (C and D) CHB hearts were first incubated with anti-CD68 (A and C) or anti-SMA (B and D). Tissue was then stained with peroxidase (brown) (A and B) or alkaline phosphatase (red) (C and D). See Figure 5 for definitions. (Original magnification × 40.)

enriched in macrophages and myofibroblasts (not shown).

### DISCUSSION

Defining the pathologic scenario that eventually results in fibrotic replacement of the AV node and, in some cases, more extensive damage to the myocardium and endocardium, has relied on in vitro studies. Histologic evaluation has been limited not only by the rarity of the disease, but also by the fact that death, if it occurs at all, most often happens weeks to months after bradycardia is first identified.

The present study is the first to evaluate the extent of apoptosis and its relationship to immunoglobulin deposition, cellular infiltration, and fibroblast transdifferentiation in cardiac tissue from fetuses dying at various time points after the clinical diagnosis of heart block. The availability of two fetal hearts almost immediately after the diagnosis of a conduction defect facilitated a window of opportunity to capture IgG deposition in close proximity to apoptotic cells and macrophages. The accumulation of these inflammatory cells and the transdifferentiation of fibroblasts into myofibroblasts (the cellular engines driving calcification and fibrosis) were prominent features in the fetuses that died earliest after diagnosis. That fibrosis was already present by the time heart block was documented strongly suggests that the cascade leading to scarring proceeds rapidly and that

reversing it will be difficult. The study of cardiac tissue from a fetus dying months after the initial diagnosis of bradycardia provides insights into the sustained nature of the disease process. Persistence of the myofibroblasts may contribute to the postnatal progression of disease in the cases of incomplete blocks at birth (11), as well as to the development of endocardial fibroelastosis (12) and life-threatening cardiomyopathies (13).

The exaggerated apoptosis observed in the hearts from fetuses with CHB/myocarditis is consistent with the notion that this specialized form of cell death plays a key role in the initiation of an inflammatory response. The highest levels of apoptosis were observed in septal regions containing conduction tissue. One plausible explanation for this increased apoptosis is derived from *in vitro* studies demonstrating that macrophages secrete cytokines such as TNF and TGF following phagocytosis of opsonized cardiocytes (4,6). Recent studies have demonstrated that TNF and TGF contribute to cell-cycle regulation of cells derived from mesenchymal lineage, indicating that these cytokines may promote apoptosis of human fetal cardiocytes in CHB (14,15). These observations support the notion that one candidate fetal factor conferring increased susceptibility to permanent cardiac injury might relate to exaggerated apoptosis, perhaps via increased secretion of TNF. This is supported by preliminary data demonstrating an increased frequency of the high-secreting -308A allele (TNF2) of TNF in children with neonatal lupus and their healthy siblings compared with that in population controls (16).

The finding of IgG in close proximity to the apoptotic cells extends previous research demonstrating that transplacentally trafficked anti-SSB/La antibodies bind apoptotic cells in the murine fetal heart (17). However, in the present study, it is fully acknowledged that the IgG specificity is unknown and awaits identification by anti-idiotypes, an approach which must take into account the possibility of private idiotypes. Although apoptosis had not been previously examined, earlier studies of fetuses dying of CHB and hydrops (at 29 and 30 weeks of gestation) showed IgG deposition in several areas of the heart including the conduction system (18,19). The investigators reported that, in some areas, "IgG appeared to outline cells" (19). In a study of endocardial fibroelastosis, Nield et al (12) demonstrated IgG deposition in the RV, LV, and AV nodal region in several cases of CHB. However, in a fetus with endocardial fibroelastosis without CHB whose mother was anti-SSB/La positive, apoptosis was not identified (20). Horsfall et al (21) demonstrated IgG binding to the surface of

myocardial fibers in a fatal case of CHB and further identified the target antigen as SSB/La using a maternal anti-SSB/La idiotype.

The notion of an inflammatory component in the cascade to cardiac fibrosis was supported by the demonstration of macrophages and multinucleated giant cells, and this finding extends the previous report of a mononuclear cell infiltration in the myocardium of a fetus dying *in utero* at 18 weeks of gestation (22), as well as the previously reported demonstration of patchy lymphoid aggregates throughout the myocardium of an infant delivered at 30 weeks and dying in the immediate postnatal period (19). Macrophages potentially contribute to several aspects of the pathologic process mediated by maternal autoantibodies. Although the pathways of clearance and cytokine secretion may vary, macrophages phagocytose both nonopsonized and opsonized apoptotic cells, the former via phosphatidylserine receptors, which is generally considered a noninflammatory process (23). In CHB, macrophages may provide a critical link between inflammation and ultimate scarring by secretion of APs resulting in increased calcification (24). In fact, macrophages could be seen contained in areas of calcification, particularly in the early cases. However, in the full-term neonate who died at birth, macrophages were less abundant and not associated with calcified areas, suggesting a diminished role in inflammation as the pathologic cascade progresses.

Abnormal stimulation of the resident fibroblasts by macrophages probably constitutes a means by which fibrotic sequelae are further amplified. Indeed, the histologic sections demonstrated the novel finding of macrophage clusters in close proximity to myofibroblasts in scar tissue near the AV groove as well as the thickened fibrous subendocardium. The functional implication of this cellular colocalization is suggested by *in vitro* studies in which cultured human fetal cardiac fibroblasts, exposed to supernatants obtained from macrophages incubated with opsonized apoptotic cardiocytes, had markedly increased expression of the myofibroblast marker SMA (scarring phenotype) (4). The addition of neutralizing anti-TGF antibodies to the "opsonized" supernatant blocked expression of SMA, supporting a potential role of TGF in the final pathologic cascade to scarring. Of relevance, preliminary genotyping data suggest that children with CHB have a higher frequency of the fibrosis-promoting polymorphism at codon 10 of TGF than do their unaffected siblings (16).

The unambiguous demonstration of macrophages and myofibroblasts in all the affected cases

substantiates the pathologic crosstalk described in reports of our earlier *in vitro* studies (4). The fibrosis observed was not bland, but involved infiltration of activated myofibroblasts as long as 5 months after the initial insult. The dedifferentiation of fibroblasts to myofibroblasts is associated with scar formation. Fibrosis is due to a persistent myofibroblast, a phenotype associated with "wounding." While it is often assumed that fibrosis is simply the end result of an inflammatory insult, a recent report of Lyme carditis with second-degree heart block (25) prompts a reappraisal of the elements of tissue injury, response, and ultimate repair or scar in the human heart. Right ventricular biopsy revealed mononuclear cells around the myocardial microvasculature and within the endocardium. Despite prolonged inflammation (heart block present for 8 weeks), the cascade to fibrosis was not irrevocably programmed, since sinus rhythm was restored following antibiotic therapy. This absence of permanent injury stands in strong contrast to the rapid progression to scarring seen in autoantibody-associated CHB. The expression of specific combinations of cytokines may ultimately provide the explanation.

In summary, immunohistologic findings in available cardiac sections from several cases of CHB/myocarditis with varying degrees of pathology parallel the results obtained exploiting *in vitro* coculturing systems. Physiologic apoptosis may initiate an inflammatory process via antibody binding and ingestion by macrophages, which not only fuels continued apoptosis but also contributes to the transdifferentiation of cardiac fibroblasts to a scarring phenotype. Persistence of this phenotype even after birth may be related to the progression of block seen in some infants postpartum. The heart block of neonatal lupus is not only progressive (second to third degree) but also characteristically irreversible, despite brief exposure to autoantibodies and limited period of inflammation. Moreover, fibrosis of the AV node contradicts the paradigm that fetal wounds heal without scarring (26). Disruption of healing may involve the continued presence of myofibroblasts, a consequence of protracted stimulation from the macrophages. Irreversible fibrotic replacement of normal tissue may be unique to heart block acquired *in utero* following autoantibody-initiated inflammation. Other inflammatory stimuli, as in Lyme disease, induce transient block (25), which is evidence against the assumption that fibroblast transdifferentiation is merely a common final pathway of inflammation.

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