Autoimmune-Associated Congenital Heart Block: The Cascade from Immunologic Insult to Fibrosis

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Summary. Taken together, these in vitro *and* in vivo *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 antibodies. AV nodal cells and perhaps the working myocardium may be particularly vulnerable to the myofibroblast, a critical "fetal factor" ultimately leading to fibrosis. It seems reasonable to predict that there are both susceptibility and regulatory factors, such as fetal polymorphisms of Fc receptors and cytokines (e.g., TGFβ), each of which could influence the extent of the proposed pathologic cascade to result in permanent third degree heart block. Dissecting the individual components in this fibrotic pathway should provide insights into the pathogenesis of antibody-associated CHB and in parallel provide a rationale basis for designing treatment to prevent irreversible injury.*

This review will provide an overview of where we are today in conceptualizing the pathogenesis of congenital heart block (CHB). It will cover the target antigens and the proposed mechanisms whereby antibodies to these targets can affect injury. Emphasis will be placed on one specific hypothesis in which exaggerated apoptosis of the developing heart leads to antibody binding, inflammation, fibroblast modulation, and finally scarring. Fetal factors, including genetic predisposition and candidate polymorphisms evaluated to date, will be briefly discussed.

Previously identified and novel candidate target antigens. The SSA/Ro and SSB/La candidate antigens and their cognate antibodies have been extensively characterized at the molecular level. Initial cloning of 60 kD Ro identified a zinc finger and an RNA-binding protein consensus motif (1-4). It has been suggested that 60 kD Ro may function as part of a novel quality control or discard pathway for 5S rRNA production (5). Anti-SSB/La antibodies recognize a 48 kD polypeptide that does not share antigenic determinants with either 52 kD or 60 kD Ro (6, 7). SSB/La facilitates maturation of RNA polymerase III transcripts, directly binds a spectrum of RNAs, and associates at least transiently with 60 kD Ro (8, 9).

In addition to the well-characterized 60 kD Ro and 48 kD La autoantigens, another target of the autoimmune response in mothers whose children have CHB is the 52 kD Ro protein (10). The full-length protein, 52α, has three distinct domains: an N-terminal region rich in cysteine/histidine motifs containing two distinct zinc fingers; a central region containing two coiled coils with heptad periodicity, one being a leucine zipper; and a C-terminal "rfp-like" domain (11, 12). Analysis by either SDS-immunoblot of human cell lines, or ELISA, reveals that between 75% and 100% of sera obtained from mothers whose children have CHB are reactive with recombinant 52 kD Ro (13-17). We have identified an alternative 52 mRNA transcript derived from the splicing of exon 4 encoding aa168-245 inclusive of the leucine zipper, which results in a smaller protein (52 ß) with a predicted mw 45,000 (18). Since expression of the alternative product 52 β is maximal at the time of cardiac ontogeny when maternal antibodies gain access to the fetal circulation, just prior to the clinical detection of bradyarrhythmia, a role for 52 β in the development of CHB is implicated (19).

Boutjdir *et al.* (20) have demonstrated that affinity-purified anti-52 kD SSA/Ro antibodies induce atrioventricular (AV) block in an isolated human fetal heart and inhibit inward calcium fluxes through L-type calcium channels in human fetal ventriculocytes (whole cell and single channel). While these observations support that maternal antibodies perturb ion flux across the cardiocyte membrane and as such may be an important contributing factor in CHB, a molecular basis has yet to be fully defined (e.g., definitive crossreactivity of anti-SSA/Ro-SSB/La with calcium channel receptor), particularly with regard to inflammation and subsequent fibrosis.

Crossreactivity between one or any of the SSA/Ro-SSB/La components and a cardiac receptor may provide a molecular explanation for pathogenicity. This hypothesis was supported by a recent report that antibodies reactive with the serotoninergic 5-hydroxytryptamine (5-HT)$_{4A}$ receptor, cloned from human adult atrium, also bind 52 kD SSA/Ro (21). Moreover, affinity-purified 5-HT$_4$ antibodies antagonized the serotonin-induced L-type calcium

channel activation in human atrial cells. Two peptides in the C terminus of 52 kD SSA/Ro, aa 365-382 and aa 380-396, were identified that shared limited homology with the 5-HT$_4$ receptor. The former was recognized by sera from mothers of children with neonatal lupus (NL) and it was this peptide that was reported to be cross-reactive with peptide aa165-185, derived from the second extracellular loop of the 5-HT$_4$ receptor [21]. Following this exciting lead, our laboratory addressed whether the 5-HT$_4$ receptor is a target of the immune response in mothers whose children have CHB [22]. Initial experiments demonstrated mRNA expression of the 5-HT$_4$ receptor in the human fetal atrium. Electrophysiologic studies established that human fetal atrial cells express functional 5-HT4 receptors. Sera from 116 mothers enrolled in the Research Registry for NL (23) and whose children have CHB were evaluated. Ninety-nine (85%) of these maternal sera contained antibodies to SSA/Ro, 84% of which were reactive with the 52 kD SSA/Ro component by immunoblot. Of the total 116 sera, none were reactive with the peptide spanning aa 165-185 of the serotoninergic receptor when background reactivity with albumin was eliminated (22). Accordingly, these results suggest that antibodies to the 5-HT$_4$ receptor do not contribute to the pathogenesis of CHB, although exchange of antisera between laboratories is underway.

Bringing the intracellular antigen to maternal antibody: the role of apoptosis. Although there are several promising new leads with regard to the target cardiac antigen(s), the initially implicated antibody-antigen pairs remain the strongest candidates at present. Accordingly, focus remains on elucidating the accessibility of these intracellular antigens to their cognate extracellular antibodies. To account for the accessibility of intracellular location and the vulnerability of the fetal heart *per se*, apoptosis in the target tissue seemed a reasonable consideration. Indeed, Tran *et al*. (24, 25) have confirmed that apoptosis occurs during the development of the murine heart. In these apoptotic cells SSB/La was translocated to the cell surface and bound by anti-SSB/La antibodies.

We have extended the murine work to the human and addressed apoptosis using cultured human fetal cardiomyocytes. Incubation of 4-day-cultured human fetal cardiocytes with 0.5 μM staurosporine or 0.3 mM 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) induced the characteristic morphologic changes of apoptosis, internucleosomal cleavage of DNA and the signature 85 kD cleavage fragment of poly ADP-ribose polymerase (PARP) (26, 27). Apoptosis could also be induced by culturing the cells on poly(2-)hydroxyethylmethacrylate (pHEMA) (28). The cellular topology of SSA/Ro and SSB/La was evaluated with con-

focal microscopy and determined in non-apoptotic and apoptotic cardiocytes, permeabilized and nonpermeabilized, by indirect immunofluorescence using two previously characterized antisera (one monospecific anti-SSB/La, and the other recognizing both 52 kD and 60 kD Ro). In non-apoptotic cardiocytes, SSA/Ro was predominantly nuclear with minor cytoplasmic staining, SSB/La was confined to the nucleus, and propidium iodide (PI) strongly stained the nucleus with a weak cytoplasmic signal. In early apoptotic cardiocytes, condensation of the PI- and SSA/Ro- or SSB/La-stained nucleus was observed, accompanied in some cases by a rim of green fluorescence (indicating SSA/Ro, SSB/La) around the cell periphery. In the later stages of apoptosis, the nuclear SSA/Ro and SSB/La staining became weaker. PI demonstrated nuclear fragmentation and blebs could be seen emerging from the cell surface, stained with both PI and SSA/Ro or SSB/La. Surface expression of SSA/Ro and SSB/La was unambiguously substantiated by scanning electron microscopy. Gold particles (following incubation with gold-labeled sera containing various specificities of anti-SSA/Ro-SSB/La antibodies and murine mAb to SSB/La and 60kD SSA/Ro) were consistently observed on early and late apoptotic cardiocytes. No particles were seen after incubation with control antisera. These data support that induction of apoptosis in cultured human fetal cardiac myocytes results in translocation of SSA/Ro and SSB/La to the cell surface, which facilitates recognition by circulating maternal antibodies.

However, it is readily acknowledged that the specificity of these particular antibodies for cardiac damage remains to be accounted for. Perhaps other autoantigens which do translocate to the apoptotic blebs (29) are not truly accessible on the surface. If intracellular trafficking of sequestered nuclear antigens to the membrane surface is not universally applicable to all such antigens, this may help explain the specificity of one antibody system versus another in the pathogenesis of disease. In support of this notion is the report by Dieude *et al*. (30) and our observation that lamin B1 is redistributed during apoptosis but, unlike SSA/Ro or SSB/La, is not bound by cognate antibodies (31). In the case of lamin B1, physiologic non-inflammatory clearance of apoptotic cells should proceed uneventfully even in the presence of circulating cognate antibodies. However, in CHB, the maternal anti-SSA/Ro-SSB/La antibodies result in opsonization and inflammatory/fibrotic sequelae (see below). Even if it turns out that SSA/Ro-SSB/La are not absolutely unique in this regard, there may be other factors such as complement binding of certain antigens or degradation of antigens that facilitate clearing without further sequelae. Establishing the fact that at least one other nuclear autoantigen is not surface-

bound during apoptosis of human fetal cardiomyocytes is a step forward.

The availability of autopsy specimens from several fetuses dying with CHB supported translation of the data obtained with cell culture to the diseased tissue itself (32). Four hearts were examined for histologic evidence of apoptosis and included: a female fetus diagnosed at 22 wk with CHB and electively terminated; a 20 wk female fetus diagnosed at 18 wk with third degree block and hydrops who died within 2 wk despite several days of maternal oral dexamethasone at 4 mg/day; a female fetus who died suddenly at 34 wk and whose autopsy unexpectedly revealed pancarditis (absent heart block or infection); and a male newborn (40 wk gestation) diagnosed with an enlarged right ventricle (RV) at 19 wk and third degree block at 24 wk, who died 2 h post-delivery. Age-matched controls included hearts obtained following elective termination of three fetuses (22, 23 and 24 wk gestation) in which there was no known cardiac disease, and from a term newborn dying of noncardiac causes.

As assessed by TUNEL (FITC and immunoperoxidase detection), apoptosis was increased in available sections (including septal tissue, RV and LV) from the 20, 22 and 34 wk fetuses with CHB/myocarditis, compared to the neonate with CHB dying at birth and 22, 23 wk control hearts. Notably, apoptotic cardiocytes were not present in contiguous tracts but were diffusely scattered between nonapoptotic cells, underscoring the selectivity of this process and that healthy cells may be injured if the apoptotic cells are not physiologically cleared (see below). In the 22 wk CHB heart, the apoptotic index (AI), a quantitative measure of apoptosis (expressed as [TUNEL-positive nuclei/total nuclei] x 100, where the total number of nuclei is the number of nonapoptotic nuclei plus the apoptotic [TUNEL-positive] nuclei) in the septal tissue was 34%, compared to 8% for RV and 2% for LV. In contrast, tissue from all anatomic regions of the 23 wk control heart revealed only scant TUNEL-positive cells consistent with physiologic apoptosis (AI < 1% for septum, RV and LV).

Although IgG deposition was more limited in distribution than apoptosis, it too was greater in the 20, 22 and 34 wk affected fetuses compared to the CHB neonate dying at birth. Specifically, IgG staining of the 20 and 22 wk CHB fetal hearts was evident in areas proximal to the AV groove, and in the LV of the 34-wk fetus with myocarditis. In contrast, IgG was not found in the septum of the normal 23 wk fetal heart or LV of the normal 22 wk heart.

Consequences of inadvertent opsonization of the apoptotic cardiocytes: the role of the macrophage. Having demonstrated that antibodies reactive with components of the SSA/Ro-SSB/La system bind the surface of apoptotic

human fetal cardiocytes (26) (as do murine cardiocytes in an *in vivo* model [24, 25]), coincubation with human macrophages was done to assess the functional consequences of "opsonization" (27). The Th1 cytokine, TNFα, was chosen as a readout of inflammation. Basal production of TNFα by the macrophages was 9.7 pg/ml and decreased to 3.3 pg/ml after coincubation with apoptotic cells, which was not observed in initial experiments using cardiocytes rendered necrotic after hypotonic lysis. Apoptotic cardiocytes preincubated with normal human IgG acted functionally as non-treated apoptotic cells. In contrast, when macrophages were cocultured with apoptotic cardiocytes incubated with affinity-purified antibodies to each of the components of the SSA/Ro-SSB/La complex, TNFα production was increased by 3- to 5-fold over basal levels and 10- to 14-fold over that secreted after culture with apoptotic cells alone. Non-apoptotic cardiocytes incubated with medium alone or with serum containing antibodies reactive with 48 kD SSB/La, 52 kD SSA/Ro, and 60 kD SSA/Ro did not modify the basal production of TNFα by the macrophages.

These observations suggest that physiologic apoptosis is inadvertently converted from an inert process designed to remodel developing tissue into one in which inflammation is evoked. Perhaps the triggering event is opsonization. Apoptotic cells have been regarded as immunosuppressive, since internalization of apoptotic cells by phagocytes inhibits the release of proinflammatory cytokines (33, 34). In contrast, phagocytosis of opsonized apoptotic cells has been reported to be proinflammatory (34, 35), an example of which is the observation that ingestion of apoptotic cells bound by anticardiolipin antibodies results in the release of TNFα from cocultured macrophages (35). Of relevance, two distinct pathways of phagocytosis, each controlled by different intracellular signaling cascades, have been identified (36). Type I phagocytosis follows binding to macrophage Fcg receptors and is considered to be proinflammatory, while type II, mediated by the complement receptor CR3, is not accompanied by inflammation. Our results support that nonopsonized apoptotic cardiocytes are ingested through type II phagocytosis. In contrast, release of the proinflammatory cytokine, TNFα, by macrophages that have ingested apoptotic cardiocytes bound by anti-SSA/Ro-SSB/La antibodies is consistent with type I phagocytosis.

Final stages of injury: the role of crosstalk between macrophages and fibroblasts. Since the signature lesion of CHB is fibrosis of the AV node, experiments were designed to examine the relationship between early antibody-mediated inflammatory events and the final sequelae leading to fibrosis. Perhaps CHB occurs as 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, separate cultures of myocytes and fibroblasts isolated from human fetal hearts were established (28). Cardiac fibroblasts at passage 3-5 were routinely used in these studies. Fibroblast enrichment in the cell culture was observed to be > 90%, as assessed using mAb clone IB10 (Sigma, F-4771) which recognizes fibroblasts. In these experiments, cardiocytes were rendered apoptotic by plating on tissue culture dishes coated with pHEMA for 18 h at 37ºC. Cells were retrieved and apoptosis assessed by TUNEL staining. Flow cytometry confirmed surface expression of SSA/Ro-SSB/La. The apoptotic cardiocytes were subsequently treated with either IgG fractions from a healthy donor or a patient with SLE whose serum was antinuclear antibody-positive, anti-SSA/Ro-SSB/La negative (nonopsonized), or with antibodies to components of SSA/Ro and SSB/La affinity-purified from a mother whose child has CHB (opsonized) and incubated with macrophages isolated from the peripheral blood mononuclear cells of healthy controls. Macrophages cultured with opsonized apoptotic cardiocytes expressed a proinflammatory phenotype, evidenced by a 3-fold increase in active $\alpha V\beta 3$ integrin.

The supernatants generated from the macrophage-cardiocyte cultures under varied conditions were tested for their effects on the cardiac fibroblasts. The readouts included expression of anti-α smooth muscle actin (SMAc), indicating a scarring myofibroblast phenotype, and proliferation ([H^3]-thymidine incorporation) of human fetal cardiac fibroblasts. Primary culture of human fetal cardiac fibroblasts exposed to supernatants obtained from macrophages incubated with opsonized apoptotic cardiocytes markedly increased the expression of the myofibroblast marker SMAc, while no effect was observed with "nonopsonized" supernatants. Supernatants from the macrophages incubated with opsonized apoptotic cardiocytes contained more TGFβ (651 pg/ml) compared to cells incubated with nonopsonized apoptotic cardiocytes (319 pg/ml) (3). The "nonopsonized" supernatants attenuated the baseline [H^3]-thymidine incorporation (69%, P < 0.02, N=9). In contrast, "opsonized" supernatant significantly reversed this inhibitory effect on proliferation (182%, P < 0.05, N=9). To examine the potential role of two candidate cytokines, TNFα and TGFβ, on the transdifferentiation of cardiac fibroblasts to SMAc-positive myofibroblasts by the "opsonized" supernatant, the effects of these cytokines and their neutralizing antibodies were evaluated. TGFβ1 increased expression of SMAc in parallel with the results of the opsonized supernatants. However, it decreased proliferation, an unexpected result suggesting that the supernatants likely contain more than TGFβ. In contrast, TNFα had no effect on either the expression of SMAc or proliferation. The addition of neutralizing anti-TGFβ antibodies to the "opsonized" supernatant blocked expression of SMAc but increased proliferation, while the anti-TNFα blocking antibodies did not affect either readout. Taken together, these data suggest that the effect of macrophage activation on the cardiac fibroblast may be a critical component leading to fibrosis of the AV node (and in some cases extending to other regions of the conduction system and heart).

To evaluate *in vivo* 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. As expected, myofibroblasts were located in areas of fibrosis (28, 32). Septal sections from the 22 wk CHB fetus 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. In septal tissue of the 20 wk CHB fetus, myofibroblasts were also found in the anticipated site of the AV node as well as in thickened fibrous subendocardial areas. Myofibroblasts were observed in the region of scarring in the CHB neonate dying at birth, and were also evident in the LV of the 34 wk pancarditic fetus. There was no evidence of SMAc-positive cells (other than those lining blood vessels) or fibrosis in either septal or ventricular tissue from the control 22, 23 wk abortuses and term neonate dying of noncardiac causes.

Given data from *in vitro* studies suggesting that factors secreted by phagocytosing macrophages result in the transdifferentiation of the fibroblasts, evidence for crosstalk between these cells was sought in the histologic sections. In the 20 wk CHB fetus, clusters of macrophages in close proximity to myofibroblasts were present in scar tissue near the AV groove as well as the thickened fibrous subendocardium. To further illustrate the relationship between macrophages and myofibroblasts, or between apoptotic cells and myofibroblasts, each of the four affected hearts was scored in identical regions for the proximity of the two cell types. For the 20 and 22 wk CHB hearts, there was a strong positive correlation between the absolute number of macrophages and the presence of myofibroblasts. In contrast, there was no correlation between apoptosis and myofibroblasts.

To evaluate the extent of fibrosis, cardiac sections were stained with picrosirius for detection of collagen. In both the 20 and 22 wk CHB hearts, there was extensive fibrosis in the inferior portion of the atrial wall where the AV node is likely to reside. Collagen deposition was absent

in the septal tissue of a fetal-age-matched normal control heart. In the 20 and 22 wk CHB hearts, TGFβ immunoreactivity was seen in the conduction tissue. In several sections, intense TGFβ staining was present in the extracellular fibrous matrix between SMAc-positive myofibroblasts concentrated in the adjacent subendocardium and infiltrating CD68-positive macrophages. Double-labeling revealed colocalization of TGFβ in the cytoplasm of macrophages, including multinucleate giant cells. No fibrosis or TGFβ immunostaining were seen in conduction tissue or ventricles of control hearts from 22, 23 wk abortuses (32).

Genetic considerations with regard to the proposed pathogenesis of CHB. It is clear that maternal antibodies are not sufficient to directly cause cardiac scarring and that additional maternal and fetal factors such as genetics are likely to convert predisposition to clinical expression. At present, no model of inheritance for CHB is known, making this a complex genetic problem. CHB occurs in 19% of siblings born subsequent to a CHB-affected infant [38], a rate 3000 times higher than the population prevalence (1/20,000) and ~10 times the rate of CHB in pregnancies of women with anti-SSA/Ro antibodies who have not previously had an affected child. This latter distinction implies a strong genetic effect. Driven by the proposed pathologic cascade supported by *in vitro* and *in vivo* data, we have begun an initial analysis of TGFb polymorphisms.

The human gene encoding TGFβ is on chromosome 19q13 and is highly polymorphic. Awad *et al*. (39) have identified five polymorphisms in the TGFβ gene: two in the promoter region at positions -800 and -509, one at position +72 in a nontranslated region, and two in the signal sequence at positions +869 and +915. The polymorphisms at positions +869 and +915 which change codon 10 (T→C, leucine→proline) and codon 25 (G→C, arginine→proline) are associated with interindividual variation in the levels of TGFβ production. This has clinical relevance since several animal and human studies have shown that high TGFβ producers develop significantly more lung fibrosis in response to a number of inflammatory triggers such as radiation (40), chemotherapy (41) and lung transplantation (42). The Pro25 allele is associated with lower TGFβ synthesis *in vitro* and *in vivo*, while the Arg25 allele is associated with allograft fibrosis in transbronchial biopsies when compared with controls and with nonallograft fibrosis (43). It has been reported that lung allograft recipients with the Leu10 allele produced the highest amounts of TGFβ (39), and chronic rejection after lung transplant is linked with high levels of TGFβ (43). In parallel with our hypothesis that high levels of TGFβ permit the development of CHB due to enhancement of extracellular matrix and increased fibrosis, patients with cystic fibrosis who develop rapid deterioration in lung function have an increased frequency of the Leu10 homozygosity (44).

Codons 10 and 25 of the TGFβ gene were evaluated in 88 children (40 CHB, 17 rash, 31 unaffected siblings) and 74 mothers from the Research Registry for NL (45). The TGFβ polymorphism Leu10 (associated with increased fibrosis) was significantly higher in CHB-children (genotypic frequency 60%, allelic frequency 78%) than unaffected offspring (genotypic frequency 29%, P=0.016; allelic frequency 56%, P=0.011) and controls, while there were no significant differences between controls and other NL groups. For the TGFβ polymorphism Arg25 there were no significant differences between NL groups and controls. Thus, children with CHB have a higher frequency of a genetic polymorphism in TGFβ (which could lead to its exaggerated secretion) than unaffected anti-SSA/Ro- exposed children which fits well with the histologic observations. Amplification of antibody-induced injury secondary to a genetic polymorphism that inherently leads to increased TGFβ production could be a factor relating to susceptibility.

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