Ro/SSA autoantibodies directly bind cardiomyocytes, disturb calcium homeostasis, and mediate congenital heart block

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Congenital heart block develops in fetuses after placental transfer of Ro/SSA autoantibodies from rheumatic mothers. The condition is often fatal and the majority of live-born children require a pacemaker at an early age. The specific antibody that induces the heart block and the mechanism by which it mediates the pathogenic effect have not been elucidated. In this study, we define the cellular mechanism leading to the disease and show that maternal autoantibodies directed to a specific epitope within the leucine zipper amino acid sequence 200–239 (p200) of the Ro52 protein correlate with prolongation of fetal atrioventricular (AV) time and heart block. This finding was further confirmed experimentally in that pups born to rats immunized with p200 peptide developed AV block. p200-specific autoantibodies cloned from patients bound cultured cardiomyocytes and severely affected Ca\(^{2+}\) oscillations, leading to accumulating levels and overload of intracellular Ca\(^{2+}\) levels with subsequent loss of contractility and ultimately apoptosis. These findings suggest that passive transfer of maternal p200 autoantibodies causes congenital heart block by dysregulating Ca\(^{2+}\) homeostasis and inducing death in affected cells.

Many autoimmune conditions are associated with increased risk of pregnancy complications and fetal loss. Complete congenital atrioventricular (AV) heart block develops in the fetus in 2–5% of Ro/SSA autoantibody-positive pregnancies of rheumatic women, usually between 18 and 24 wk of gestation (1, 2). Initiated as a first-degree AV block (3), the condition progresses to a complete third-degree AV block after mononuclear cell infiltration, fibrosis, and calcification of the cardiac tissue (4, 5).

The Ro/SSA antigen is intracellular and contains Ro52 and Ro60 protein components to which autoantibodies are induced in the mother (6). Systematic analyses have been undertaken to identify the subpopulation and specificity of Ro/SSA antibodies that correlate with congenital heart block (7–9). Recent studies indicate that antibodies recognizing the Ro52 protein of the Ro/SSA complex are pathogenic (3, 9), and more specifically, our studies have demonstrated that antibodies to amino acids 200–239 (p200) of the Ro52 protein were detected in the mothers of children with complete heart block (9). However, the fine specificity and the mechanism by which p200-specific antibodies mediate heart block have not been elucidated.

We and others have shown that early treatment of an incomplete block with high dose fluorinated steroids prevents progression of, or even reverts, the block, decreasing fetal morbidity and mortality (3, 10, 11). However, a complete third-degree block is permanent (11), making it relevant also from a clinical point of view to define the specific antibody-mediated heart block. A marker with high predictability could identify high risk pregnancies and allow initiation of treatment at the critical stage to prevent irreversible heart block in the fetus.

In this paper, we show that not all, but Ro52 autoantibodies with a particular specificity for the p200 sequence of the Ro52 protein correlate with AV time prolongation in the fetus, bind the surface of cardiomyocytes, and...
induce Ca\(^{2+}\) dysregulation and ultimately apoptosis in affected cells.

**RESULTS AND DISCUSSION**

Maternal anti-p200 antibody levels correlate with neonatal AV conduction time

To evaluate the role of Ro52 antibodies in development of congenital heart block, we followed 25 pregnant Ro52 autoantibody-positive women prospectively with weekly fetal echocardiographic examinations between 18 and 24 wk of gestation. Maternal autoantibodies to different parts of the Ro52 protein (Fig. 1 A) were investigated by ELISA. Fetal AV time was defined using two different Doppler techniques (Fig. 1, B and C), and development of heart block was correlated with antibody specificity. 9 of the 25 (36%) fetuses had signs of first-degree AV block by both methods. One of these nine developed a second-degree and another a complete AV block (Videos 1 and 2, available at http://www.jem.org/cgi/content/full/jem.20041859/DC1). We found a significant correlation between prolongation of AV time and levels of antibodies to amino acids 200–239 (p200) of Ro52 (P < 0.02). Mothers of fetuses developing second- and third-degree AV block were found among those with the highest levels of p200 antibodies (Fig. 1, D and E). In mothers of less affected fetuses, the Ro52 antibody response was mainly directed to the p176 peptide (amino acids 176–196) of the Ro52 protein, and interestingly, the ratio of p200/p176 antibody levels correlated more significantly with AV time prolongation (P < 0.005; Fig. 1, F and G).

p200 immunization of rats leads to AV block in the pups

To directly test whether antibodies to the p200 amino acid stretch of Ro52 were responsible for development of heart block, we immunized female DA rats with p200 or a control peptide. A specific antibody response to p200 peptide and full-length Ro52 protein developed in p200-immunized animals, but not in control immunized animals (Fig. 2, A and B). The antibodies also bound the overlapping peptide p197, but there was no response to any of the other Ro52 peptides, suggesting that no epitope spreading to adjacent peptides occurred (Fig. 2 C). The antibodies to p200 were mainly of IgG2a, IgG2b, and IgG2c subclasses (Fig. 2 D). The animals were boosted several times, and when stable anti-p200 titers were observed, rats were mated and the effects on the cardiac conduction system in the offspring were evaluated by electrocardiogram (ECG) within 12 h after birth (Fig. 2, E and F). About 20% (10/52) of the pups exposed to anti-p200 antibodies during their fetal life developed a first-degree AV block (Fig. 2 G), whereas none of the pups from control immunized mothers had heart block.

A similar epitope is recognized by antibodies from children with congenital heart block, rat pups with AV block, and a human p200 monoclonal

To generate a monospecific source of human p200 antibodies, we screened phage display libraries derived from peripheral B cells of Ro52 antibody-positive patients (12). Two clones, denoted S3A8 and M4H1, with binding specificity for Ro52 and p200 were isolated by this technology. A set of mutated p200 peptides was designed to determine the fine epitope specificity of the two human monoclonal antibodies and to explore possible structural as well as linear epitopes within the \(\alpha\)-helical p200 fold (Fig. 3 A). The peptide pZIP was designed to create an optimal leucine zipper with high dimer stability (Fig. 3 B), thus inhibiting binding to the dimer interface. In pOUT, negatively charged amino acids on the outer surface of the predicted zipper were substituted for positively or uncharged residues to alter the antigenicity while maintaining an intact structure. The dif-
The horizontal line marks the mean PR interval of the 26 pups from control rats was analyzed and 10 (19%) had a first-degree AV block (filled symbols).

1. **Figure 2.** Immunization of rats with p200 peptide leads to AV block in the offspring. Rats immunized with p200 developed an antibody response against p200 (A) and full-length Ro52 (B), whereas rats immunized with control peptide did not. (C) No epitope spreading to adjacent Ro52 peptides was observed in p200-immunized animals, although to some extent antibodies did bind to the highly overlapping p197 peptide. (D) The induced anti-p200 antibodies were mainly of IgG2a, IgG2b, and IgG2c subclasses in mothers as well as pups. (E) Immunized rats were mated and antibody titers and AV time were monitored in the pups by ECG performed on all pups within 12 h after birth. HR, heart rate. ECGs were sampled for 5 s four times per minute and the PR interval was calculated from an averaged complex (F). Arrows denote start and end points for measurements on the averaged ECG. (G) ECG from a total of 52 pups from p200-immunized rats was analyzed and 10 (19%) had a first-degree AV block (filled symbols). The horizontal line marks the mean PR interval of the 26 pups from control immunized animals (mean ± SD; 58.9 ± 3.2).

2. p200 antibodies bind the cell surface and dysregulate calcium homeostasis in cardiomyocytes, leading to cell death

Our results from both human and animal studies indicate that antibodies to p200 are involved in the development of congenital heart block. However, whether p200-specific antibodies can bind to cardiomyocytes and induce pathogenic effects has not been addressed. To directly investigate this issue, we established primary cardiomyocyte cultures from neonatal rat hearts. First, cell surface binding was examined and S3A8, but not the M4H1, monoclonals displayed binding (Fig. 4 A). Ca$^{2+}$ is one of the main regulators of cardiomyocyte pacemaking and contractility, and to evaluate the effect of p200 autoantibodies on cardiomyocyte function we measured Ca$^{2+}$ oscillations using Fluo-4 dye under a laser confocal microscope at steady-state level and after the addition of antibody.

S3A8, but not M4H1 or vehicle (PBS), had a profound effect on oscillations and survival of the cardiomyocytes. When S3A8 was added to spontaneously contracting and oscillating cardiomyocytes, we noted an initial phase of increased frequency of Ca$^{2+}$ transients. This was followed by a progressive decrease in frequency of Ca$^{2+}$ oscillations, resulting in accumulation of intracellular Ca$^{2+}$, loss of Ca$^{2+}$ oscillations, and contractility and subsequent cell death of the cardiomyocytes (Fig. 4, B–F). After 1 hr, no cells exhibiting Ca$^{2+}$ oscillations or contractions were observed in S3A8-treated cardiomyocytes (Fig. 4 D), and after 24 h, no contracting cells were observed. In contrast, cells in cultures treated with M4H1 antibody still exhibited a similar Ca$^{2+}$ load and oscillating and contracting activity with unchanged frequency at both 60 min and 24 h of treatment (Fig. 4, D–F). Cellular Ca$^{2+}$ overload triggers apoptosis (13), and cell death via this pathway in S3A8-treated cells was confirmed by caspase-3 and TUNEL staining (not depicted). Quiescent cells not displaying spontaneous oscillations were also induced by S3A8 treatment to begin Ca$^{2+}$ oscillations with Ca$^{2+}$ transients of increasing frequency and subsequent Ca$^{2+}$ accumulation (Video 3 and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20041859/DC1).

Sera from p200-immunized rats had effects similar to S3A8. With sera from two different p200-immunized ani-
mals, an initial increased Ca\(^{2+}\) frequency oscillation activity was noted (Fig. 4 D). Most cells in cultures treated with anti-p200 sera had lost all Ca\(^{2+}\) oscillation activity after 60 min and showed intracellular Ca\(^{2+}\) accumulation (Fig. 4, E and F), though a few cells oscillating at low frequency still remained. These cells were also active at 24 h of examination. The effects were not observed with control sera from two control peptide–immunized animals (not depicted).

These studies demonstrate that anti-Ro52 antibodies with specificity for the p200 epitope confer the pathogenic effects during development of congenital heart block and might be used as a serologic correlate for the development of heart block. However, our data suggest that not all anti-p200 antibodies are pathogenic. We believe that anti-p200 antibodies with the fine specificity profile of S3A8 constitute the pathogenic antibodies, as these antibodies directly bind cardiomyocytes, alter Ca\(^{2+}\) homeostasis, and eventually lead to cell death by apoptosis. We suggest that these pathogenic effects are not caused by interaction with Ro52, as this protein is intracellular, but that the antibodies are potentially binding a cross-reactive self-antigen on the cell surface of cardiomyocytes. Apoptosis has been shown to occur in congenital heart

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Figure 3. An epitope within the predicted leucine zipper structure is recognized by similar antibodies from congenital heart block children, rat pups, and selected human anti-p200 monoclonals. (A) Ribbon structure representation of the predicted p200 secondary structure fold, including the leucine zipper motif. Leucines are represented in gray. (B) Schematic drawing of the region around the predicted leucine zipper and mutated peptides used in epitope mapping of anti-p200 antibodies. The amino acid sequence of p197 and p200 (leucines in gray and underlined) are shown, as well as indications of amino acid substitutions in the mutated peptides (pZIP in blue and in pOUT in red). The antigenic contribution from the COOH-terminal amino acid was investigated by an alanine scan of amino acids 233–239. S3A8 (C) and M4H1 (D) binding to the peptides was investigated. Sera from children with congenital heart block (E) and pups from p200–immunized animals with first-degree AV block (F) contained mainly S3A8 idiotype-like p200-specific antibodies. The level of p200 reactivity in each monoclonal or individual was set as 1 and the values for each monoclonal/individual normalized against their respective p200 reactivity is shown.
block–affected fetal hearts (5), and it has been suggested that Ro52-antibodies bind to the Ro52 protein exposed on the surface of naturally occurring apoptotic cells of the developing heart. Our results, however, demonstrate that the Ro52 antibodies of p200 specificity in fact induce the apoptotic process by causing Ca$^{2+}$/H$^{+}$ overload, thereby potentially initiating the whole process of heart block development. In conclusion, multiple lines of investigation presented in our paper including data from patients, an animal model, and in vitro studies, all indicate that antibodies of S3A8-like p200 specificity initiate heart block development by dysregulating Ca$^{2+}$ homeostasis. We suggest that this antibody specificity is the essential and initiating factor in the development of congenital heart block and that it could be used clinically as a tool to identify high risk pregnancies, thereby enabling early treatment and prevention of congenital block.

Figure 4. S3A8 anti-p200 monoclonals and sera from p200–immunized rats, but not M4H1, p200 monoclonals, or vehicle bind cardiomyocytes and induce dysregulation of Ca$^{2+}$/H$^{+}$ homeostasis. (A) Primary cardiomyocytes prepared from neonatal rat hearts were stained with S3A8, M4H1, or PBS. (B) Primary cardiomyocyte cultures loaded with fluo-4 before the addition of monoclonal antibodies or serum. (C) Single cell tracing of [Ca$^{2+}$]$_i$ (calcium dye fluorescence intensity in arbitrary units) after the addition of respective antibody or vehicle. (D) The mean relative frequency of Ca$^{2+}$ transients. (E) Change in baseline intracellular Ca$^{2+}$/H$^{+}$ levels over time. ΔF/F$_0$ was calculated by dividing baseline fluorescence between transients by the original (time 0) baseline level. (D and E) A representation of results from 10 independent experiments for S3A8, 6 independent experiments for M4H1, 5 independent experiments for PBS, and 2 experiments with 2 different rat sera (1:10). Each rat serum is depicted individually (D and E). (F) Baseline fluorescence intensity after 20 min of substance application is depicted. A scale of 0–250 arbitrary units was used, where intensity of average pixel fluorescence signal is saturated when color turns blue (>$250$ arbitrary units).
MATERIALS AND METHODS

Patients. 25 pregnant anti-Ro52\(^+\) women were included consequentially upon referral to the Pediatric Cardiology Unit at Karolinska Hospital for monitoring during pregnancy (Table S1, available at http://www.jem.org/cgi/content/full/jem.20041859/DC1). The study was approved by the Stockholm North Ethics Committee and was in accordance with the Declaration of Helsinki. The mothers were mated 2–4 wk after the last booster. The Stockholm North Ethics Committee approved the study.

ELISA for detection of antibodies to Ro52, Ro60, La, and Ro52 peptides. ELISA for human sera was performed as described previously (9). Sera were tested at a 1:1,000 dilution. Rat sera were analyzed at 1:2,500, and bound antibodies were detected using rabbit anti-rat IgG, IgG1, IgG2a, IgG2b, or IgG2c (Nordic).

Immunization and ECG recordings in rats. 6-wk-old female DA rats (B&K) were immunized with p200 or virally derived control JB4 peptide (amino acid sequence G1WGC6GKLICCTAVWPNAS; reference 15). Rats were mated 2–4 wk after the last booster. The Stockholm North Ethics Committee approved the study.

Expression of scFv antibodies. Expression and purification of scFv antibody fragments were performed as described previously (12). The purified antibodies were dialyzed against several changes of PBS and filtered for sterility before use.

Preparation of primary cardiomyocyte cell cultures from rat pups. Cultures of cardiomyocytes were prepared using a kit (Worthington Biochemical Corporation). Hearts from 1-d-old DA rats were dissected and prepared according to the manufacturer’s instructions. The cardiomyocytes were cultured in DMEM/F12 supplemented with 10% FCS, 1 µg/ml gentamycin, 2.5 µg/ml insulin, 2.5 µg/ml transferrin, 2.5 ng/ml selenium, 30 µg/ml BrdU, and 15 mM Hepes at 37°C with 5% CO\(_2\).

Calcium level measurements. Cardiomyocytes prepared as described above were cultured on polystyrene-covered glass slips (VWR) for 5 d. Cells were loaded with the Ca\(^2+\) indicator fluo-4 acetoxymethyl ester (fluo-4 a.m.; Molecular Probes) by incubation for 50 min at 37°C in 5% CO\(_2\), in conditioned medium containing 2 mM fluo-4 a.m. mixed with pluronic acid (final concentration: 0.2%). This was followed by 20 min of deesterification before measurements began. The coverslips were mounted in a chamber with conditioned medium (37°C) and analyzed with an inverted confocal microscope (TCS SP; Leica). 20 min of consecutive images collected every 1.705 s (and in some cases every 0.7 s) were recorded for each experiment. Cells were returned to the incubator and reexamined 60 min and 24 h after drug application.

Images were processed with the ImageJ software (NIH) and imported into Microcal Origin 7.5 (Originlab.com) for further analysis.

Statistical analysis. The Mann-Whitney U test was used to compare autoantibody levels between pregnant women with and without fetal AV block. A p-value of <0.05 was considered significant.

Online supplemental material. Video 1 illustrates echocardiographic recordings from a fetal heart with signs of first degree AV block, and in Video 2, the complete third-degree AV block after progression in the same patient is shown. Fig. S1 contains explanatory anatomical labels for Video 1 and 2. Video 3 contains a film recorded in the confocal microscope of fluo-4-loaded cardiomyocytes before and after the addition of S3A8 antibody, and in Fig. S2 a single cell tracing of [Ca\(^{2+}\)]\(_e\), from this experiment is shown. Table S1 contains information on the patients. Videos 1–3, Figs. S1 and S2, and Table S1 are available at http://www.jem.org/cgi/content/full/jem.20041859/DC1.

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