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Beta2-glycoprotein-I and Protection from Anti-SSA/Ro 60 Associated Cardiac Manifestations of Neonatal Lupus

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Abstract

One mechanism to molecularly explain the strong association of maternal anti-Ro60 antibodies with cardiac disease in neonatal lupus (NL) is that these antibodies initiate injury by binding to apoptotic cardiomyocytes in the fetal heart. Previous studies have demonstrated that beta2-glycoprotein I (β_2GPI) interacts with Ro60 on the surface of apoptotic Jurkat cells and prevents binding of anti-Ro60 IgG. Accordingly, the current study was initiated to test two complementary hypotheses a) competition between β_2GPI and maternal anti-Ro60 antibodies for binding apoptotic induced surface translocated Ro60 occurs on human fetal cardiomyocytes and b) circulating levels of β_2GPI influence injury in anti-Ro60 exposed fetuses. Initial flow cytometry experiments conducted on apoptotic human fetal cardiomyocytes demonstrated dose-dependent binding of β_2GPI. In competitive inhibition experiments, β_2GPI prevented opsonisation of apoptotic cardiomyocytes by maternal anti-Ro60 IgG. ELISA was used to quantify β_2GPI in umbilical cord blood from 97 neonates exposed to anti-Ro60 antibodies, 53 with cardiac NL and 44 with no cardiac disease. β_2GPI levels were significantly lower in neonates with cardiac NL. Plasmin-mediated cleavage of β_2GPI prevented binding to Ro60 and promoted the formation of pathogenic anti-Ro60 IgG-apoptotic cardiomyocyte complexes. In aggregate these data suggest that intact β_2GPI in the fetal circulation may be a novel cardioprotective factor in anti-Ro60-exposed pregnancies.

Introduction

The identification of isolated congenital heart block in utero during the mid to late second trimester is almost universally associated with maternal Abs to a component of the SSA/Ro-SSB/La ribonucleoprotein complex, even in asymptomatic women. The cardiac disease of neonatal lupus (cardiac NL), while typically characterized by fibrosis of the atrioventricular node, can extend to the working myocardium and endocardium (1). Although the accessibility of maternal Ab to a normally sequestered intracellular antigen has been difficult to reconcile, apoptosis has been proposed as a cellular event which promotes the

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translocation of Ro and La proteins to the cell surface and binding by cognate Abs (2, 3). This notion led to the observation that healthy cardiomyocytes are capable of engulfing apoptotic cardiomyocytes and that binding of anti-Ro/La Abs to the apoptotic cardiomyocytes inhibits this physiologic process (4). Histological studies support the in vitro findings since hearts from fetuses dying with cardiac NL reveal exaggerated apoptosis, while apoptosis is rarely detected in healthy hearts from electively terminated age matched fetuses (5).

The direct pathogenicity of maternal anti-Ro60 Abs has been questioned since cardiac NL occurs in only 2% of neonates born to mothers with the candidate Abs (1). Although Abs appear necessary, it is likely that fetal and environmental factors amplify the Ab effect to promote full expression of disease. A focus on beta$_2$-glycoprotein I (β_2GPI) as a candidate fetal factor is supported by two recent observations a) Ro60 expressed on the surface of apoptotic Jurkat cells interacts with β_2GPI and b) preincubation of the apoptotic cells with β_2GPI significantly blocks the binding of anti-Ro60 Abs (6, 7). β_2GPI is an abundant positively charged protein comprised of five short consensus repeats with a lysine patch adjacent to a hydrophobic C-terminal loop (residues 313–316) in domain V (8). Significantly lower levels of circulating β_2GPI were reported in umbilical cord plasma compared to adult plasma (9) which may be relevant to the clinical observation that the maternal heart is not affected despite continuous exposure to the identical Abs. β_2GPI has been implicated in the modulation of coagulation and fibrinolysis pathways (10) and is regulated by plasmin which proteolytically cleaves β_2GPI domain V (11, 12), the putative site for binding by Ro60 (6). Of further relevance to the pathogenesis of cardiac NL, the binding of anti-Ro60 IgG to apoptotic cardiomyocytes was recently shown to enhance the activity of urokinase plasminogen activator (uPA) which catalyses the conversion of plasminogen to plasmin (13). This may in turn result in an amplification cycle whereby anti-Ro60 binding results in increased plasmin generation, cleavage of β_2GPI and further uncompeted binding by pathogenic Ab.

Accordingly, this study was initiated to evaluate the hypothesis that in utero levels of β_2GPI influence pregnancy outcome in anti-Ro60-positive mothers. The relevance of the Ro60-β_2GPI interaction to the pathogenesis of cardiac NL was approached by using the target cell, human fetal cardiomyocytes, and affinity purified anti-Ro60 IgG from a mother of an affected child. The levels of β_2GPI were measured by ELISA in umbilical cord blood from affected and unaffected neonates, each exposed to maternal anti-Ro60 Abs. The Ro60-β_2GPI binding site was then mapped to determine whether cleavage of β_2GPI by plasmin, affects binding to Ro60.

Materials and Methods

Patients and Controls

Umbilical cord serum or plasma from neonates of anti-Ro60-positive mothers and serum from mothers were obtained, with informed consent. Pregnancies resulting in cardiac NL or no cardiac manifestations were identified from the Research Registry for Neonatal Lupus (RRNL) (14) and PR Interval and Dexamethasone Evaluation (PRIDE) (15). Each database has IRB approval for evaluation of de-identified information. Maternal serum and cord blood contained Abs reactive with Ro60 and/or Ro52 and La as measured by an NYU CLIA approved laboratory. Total IgG and affinity-purified Abs against recombinant Ro60 (16) were prepared from maternal serum (4). Anti-β_2GPI Abs were measured in maternal sera by QUANTA Lite® β_2GPI IgG ELISA (INOVA Diagnostics, San Diego, CA).

Induction of apoptosis in cultured human fetal cardiomyocytes and Jurkat cells

Healthy fetal hearts of gestational ages 16–24 weeks were obtained after elective termination and obtaining consent from the mothers through Dr. Brad Poulos at the Human Fetal Tissue Repository of Albert Einstein College of Medicine in accordance with the guidelines of the Institute Review Board of New York University School of Medicine. Cells were isolated and cultured as described (4). Apoptosis was induced with 1 μg/ml staurosporine for 4 h in serum free DMEM or by treating cardiomyocytes with IFN-γ for 24 h and plating on poly-HEMA in the presence of IFN-γ, TNF-α, and cycloheximide (4). Early and late apoptotic Jurkat cells were prepared as described (17). Apoptosis was confirmed by flow cytometric analysis of phosphatidylserine exposure (Annexin V binding).

Measuring β_2GPI binding to apoptotic cardiomyocytes and competitive inhibition of anti-Ro60 IgG

Native purified human β_2GPI (Haematologic Technologies, Essex Junction, VT) was added to apoptotic cardiomyocytes or Jurkat cells for 30 min at room temperature, washed and detected by either polyclonal rabbit anti-β_2GPI antiserum or anti-β_2GPI domain I mAb (a gift from Professor Steven Krilis, University of New South Wales, Australia) (18) by flow cytometry (6). A proteolytically clipped form of β_2GPI was generated with human plasmin (Sigma, St. Louis, MO) (19) and binding to apoptotic cells was determined (6). In competitive inhibition experiments, increasing concentrations of β_2GPI (0.5–50 μg/ml) or IgG depleted-cord blood (diluted 1/10) were co-incubated with 10 μg/ml affinity-purified anti-Ro60 IgG or healthy control IgG in the presence or absence of plasmin (0.2–3 μg/ml) and binding assessed with an anti-human IgG-FITC by flow cytometry (17). To confirm the specificity of the β_2GPI mediated inhibition of anti-Ro60 IgG binding to apoptotic cardiomyocytes, recombinant human Growth Arrest Specific 6 (GAS6) or milk fat globule-EFG factor 8 (MFG-E8) were used in competitive inhibition experiments as described above. Binding of GAS6 and MFG-E8 to apoptotic cardiomyocytes was detected with an anti-penta-His mAb (Qiagen) that recognizes the C-terminal 6-His tag in both proteins.

Measuring β_2GPI levels in umbilical cord blood by ELISA

β_2GPI levels in umbilical cord serum or plasma were determined by a capture ELISA adapted from McNally and colleagues (20). Briefly, rabbit polyclonal anti-β_2GPI antiserum diluted 1/2000 in PBS was coated onto 96-well Nunc Maxisorp™ microtitre plates. All incubations were conducted at 37°C for 1 h. Wells were blocked with 2% BSA/PBS and then washed 3 times with PBS containing 0.05% Tween 20. A standard curve was constructed with known concentrations of purified human β_2GPI. In some experiments plasmin cleaved β_2GPI was added to ELISA plates. Umbilical cord serum or plasma were diluted 1/1000, 1/2000, and 1/4000 in 1% BSA/PBS. After extensive washing, anti-β_2GPI mAb was applied and detected with alkaline phosphatase conjugated anti-mouse IgG (Sigma).

Assessing β_2GPI levels in umbilical cord blood by Immunoblot

To confirm ELISA findings, a panel of 9 umbilical cord bloods, 5 from neonates affected by cardiac NL and 4 non-cardiac NL neonates, were subjected to SDS-PAGE under non-reducing conditions and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat skim milk powder in PBS overnight at 4°C, washed 3 times in PBS/0.1% Tween 20 and probed with either rabbit anti-β_2GPI antiserum (1/2000) or mAb (0.5 μg/ml) for 1 h at room temperature. Membranes were stained with IRDye® 800CW anti-rabbit or anti-mouse IgG conjugates and analysed on the Odyssey® Infrared Imager (LI-COR® Biosciences).

Binding recombinant Ro60 to immobilized native or plasmin-clipped β_2GPI

Soluble overlapping fragments of mouse Ro60 encompassing aa 82–244, 82–146, 82–192, 149–244 and 193–236 expressed as maltose-binding protein (MBP) fusion constructs were prepared from pMAL cDNAs (a gift from Kenneth Kaufman, Oklahoma Medical Research Foundation) (21). All recombinant proteins were biotinylated with Sulfo-NHS-Biotin (Pierce, Rockford, IL) according to the manufacturer's instructions. Binding of biotinylated MBP-Ro60 aa 82–244, aa 82–146, aa 82–192, aa 149–244 or aa 193–236 to native or plasmin-cleaved β_2GPI was assessed by ELISA (6).

Molecular modelling of the Ro60-β_2GPI complex

A homology model of the 3-dimensional structure of human Ro60 was constructed based on the crystal structure of *Xenopus laevis* Ro60 using the Swiss Model Program (22). Molecular visualisation and hypothetical docking experiments of Ro60 and the crystal structure of human β_2GPI was performed using Swiss Pdb Viewer (23) and manual docking of the structures based on experimental mapping data, surface charge and stereochemical complementarity.

Statistical Analysis

Dichotomous variables (sex, race, maternal Ab status, dexamethasone use, and delivery method) between cardiac NL and non-cardiac NL neonates were compared by Fisher's exact test. Continuous variables (birth weight, gestational age, titers of anti-Ro60 Abs, and levels of β_2GPI) were compared in neonates with cardiac NL and non-cardiac NL by the unpaired T-test. Levels of β_2GPI in umbilical cord blood from twins were compared by paired T-test. Multivariate regression analysis was conducted including cardiac NL, dexamethasone use, and delivery method as predictor variables in the model. Two-sided p values <0.05 were considered statistically significant.

Results

β_2GPI binds to apoptotic cardiomyocytes and inhibits opsonisation by maternal anti-Ro60 Abs

To test the hypothesis that β_2GPI may be a protective factor in cardiac NL by preventing the formation of pathogenic anti-Ro60 IgG-apoptotic cardiomyocyte complexes in the fetal heart, initial experiments assessed the binding of β_2GPI to apoptotic human fetal cardiomyocytes. Apoptosis was induced either by staurosporine (intrinsic pathway) or a loss of anchorage in the presence of INF-γ, TNF-α, and cycloheximide (extrinsic pathway). The latter treatment induced Annexin V- positivity consistent with apoptosis in 75% of the cell population, of which 38±5% were considered early apoptotic (PI-negative) and 37±13% late apoptotic (PI- positive) (Figure 1A). Staurosporine treatment generated approximately 66% Annexin V positive cells, of which 29±18% and 37±4% were considered early and late apoptotic respectively. Purified native human β_2GPI bound to both early and late apoptotic cardiomyocytes in a saturable and dose-dependent manner, with a 2-fold increase in binding to late apoptotic cells compared to the early apoptotic population (Figure 1B). Affinity purified anti-Ro60 IgG from a mother of an infant with cardiac NL bound to both early and late apoptotic cardiomyocytes, with enhanced binding to the late apoptotic population. Increasing the concentration of β_2GPI revealed a dose-dependent inhibition of anti-Ro60 IgG binding to early and late apoptotic cardiomyocytes (Figure 1C). These findings confirm that β_2GPI competes with anti-Ro60 IgG for binding to the surface of apoptotic cells and extends the observation to human fetal cardiomyocytes. To address the specificity of the Ro60-β_2GPI interaction on apoptotic cardiomyocytes competitive inhibition experiments were repeated using GAS6 or MFG-E8, two molecules that also bind apoptotic cells (24,

25). Neither GAS6 nor MFG-E8 altered anti-Ro60 IgG binding to apoptotic cardiomyocytes (Figure 1D). Binding of GAS6 and MFG-E8 to apoptotic cardiomyocytes was confirmed with an anti-penta-His mAb (data not shown).

Circulating levels of β_2GPI are lower in cord blood from anti-Ro60 Ab exposed neonates with cardiac NL compared to non-cardiac NL neonates

Having established in vitro proof-of-concept for the protective β_2GPI hypothesis, translation to pathogenesis was sought by measurement of circulating β_2GPI levels in affected and unaffected neonates exposed to maternal anti-Ro60 antibodies. Because it is not feasible to obtain fetal blood during the critical time of heart injury (18–24 weeks gestation) umbilical cord blood was used as a proxy to assess the potential influence of circulating β_2GPI levels in the pathogenesis of disease. Umbilical cord blood (serum or plasma were previously reported to have no differences in β_2GPI levels (20, 26)) from 97 anti-Ro60 exposed infants were studied, 53 with cardiac NL and 44 with no cardiac manifestations (Table 1). Of the 53 affected children, 49 had congenital heart block (47 3rd degree and 2 2nd degree), 3 had an isolated cardiomyopathy and 1 had sustained sinus bradycardia. Of the non-cardiac NL neonates (many of whom had affected siblings), 38 were completely healthy, 5 had cutaneous-NL and 1 had hepatic/haematological manifestations of NL. The demographic characteristics, mother's antibody status and medication, method of delivery, and infant birth weight and gestational age are shown in Table 1. The sex, race, maternal antibody profile and titer of anti-Ro60 antibody were not significantly different between affected and unaffected neonates. As expected, mothers of children with cardiac NL were more likely to have taken dexamethasone and deliver by caesarean section (p=0.0001). Cardiac NL children were more frequently born prematurely and of lower birth weight than the non-cardiac NL controls (p=0.007).

β_2GPI levels as assessed by ELISA were significantly lower in the neonates with cardiac NL (133.6±7.6 μg/ml) compared to those without cardiac NL (193.5±10.6 μg/ml) (p<0.0001) (Figure 2A). Dexamethasone use and delivery status were also significantly associated with β_2GPI (p=0.05, p=0.01, respectively). Gender, race, maternal antibody status, birth weight and gestational age were not significantly correlated with β_2GPI. In a multivariable regression analysis which included cardiac NL status, dexamethasone use and delivery method as predictor variables in the model, β_2GPI levels remained lower in cardiac NL compared to non-cardiac NL (p=0.06). However, the effects of dexamethasone use and delivery method were substantially diminished and no longer significantly associated with β_2GPI levels after adjusting for cardiac NL status (p=0.53 and p=0.40, respectively). These results are likely explained by the fact that dexamethasone is generally only given to treat cardiac NL once identified, not for prophylaxis, and C-section is more common for delivery of a baby with cardiac NL than an otherwise healthy baby. Four twin pairs discordant for cardiac NL were available for study (2 monozygotic and 2 dizygotic) and proved to be highly informative. In each pair, the twin affected by cardiac NL had lower levels of β_2GPI than the unaffected twin (p=0.04) (Figure 2A). In the non-cardiac NL group, there were no significant differences between β_2GPI levels in the children affected by rash or those without any manifestation of NL (data not shown). Titres of anti-Ro60 antibodies were equivalent in the sera obtained at the time of delivery from mothers of children with cardiac NL (23,932±4,647, n=47) compared to non-cardiac NL (27,138±6,623 n=40) (p=0.69) and in the umbilical cord bloods from neonates with cardiac NL (11,814±2,021, n=47) vs. non-cardiac NL neonates (14,286±2,829 n=39) (p=0.47).

To substantiate the ELISA findings regarding β_2GPI concentration and to investigate functional relevance, a subset of umbilical cord blood from 4 unaffected controls and 5 cardiac NL children were selected for further analysis. Immunoblot results were consistent with those of the ELISA in that the density of bands was lower in the 5 umbilical cord

bloods from the neonates with cardiac NL compared to the 4 bloods from the healthy neonates (Figure 2B). Cord blood from an unaffected control (β_2GPI = 367 μg/ml) and a neonate with cardiac NL (β_2GPI = 154 μg/ml) were selected for competitive inhibition experiments to support the protective effects of higher circulating levels of β_2GPI. Prior to the experiment, IgG was removed from each cord blood by protein A to eliminate the effect of endogenous maternal anti-Ro60 antibodies. As assessed by flow cytometery, exogenously added affinity purified anti-Ro60 IgG bound at higher levels to apoptotic cardiomyocytes in the presence of cardiac NL cord blood compared to an equal quantity of cord blood from the healthy neonate (data not shown).

It is plausible that concomitant levels of maternal anti-β_2GPI antibodies may impede the protective function of β_2GPI and account for the lower levels of β_2GPI measured in umbilical cord blood from neonates with cardiac NL. To test this consideration, 40 maternal sera obtained at the time of birth (20 cardiac NL, 17 non-cardiac NL, and 3 discordant twins) were available for evaluation of antibodies to β_2GPI. Of fourteen tested in which the associated cord blood level of β_2GPI was low (<150 μg/ml), only 1 had anti-β_2GPI antibodies. Overall, only two of the 40 were positive for anti-β_2GPI antibodies (1 cardiac NL and 1 non-cardiac NL). These data suggest that maternal anti-β_2GPI antibodies are not associated with a loss of the protective function of β_2GPI or the low levels of β_2GPI observed in the cord bloods.

Molecular mapping reveals β_2GPI binds to Ro60 aa 82–146 via its domain V hydrophobic loop

Molecular studies were conducted to assess the β_2GPI-Ro60 interaction and factors that may affect this interaction and ultimately generate cardiac vulnerability. β_2GPI was previously shown to bind within Ro60 amino acids (aa) 82–244, an immunodominant region of the molecule (22, 27). To more precisely map the β_2GPI binding site on Ro60, soluble recombinant MBP fusion proteins expressing fragments of Ro60 within aa 82–244 were used in direct binding experiments. β_2GPI bound MBP-Ro60 aa 82–192 and aa 82–146, but not MBP-Ro60 aa 149–244 or aa 193–236 or MBP control (Figure 3A). These results indicate that β_2GPI binding occurs predominantly within Ro60 aa 82–146, an alpha helical region of the protein that forms a distinct cleft containing clusters of hydrophobic residues interspaced by acidic amino acids (Figure 3A). To map the β_2GPI binding site on Ro60 expressed on the surface of apoptotic cells, flow cytometry competitive inhibition experiments were performed with β_2GPI and the Ro60 sub-fragments. MBP-Ro60 aa 82–192 and MBP-Ro60 aa 82–146 reduced the binding of β_2GPI to late apoptotic Jurkat cells by 35.3±2.7% and 18.8±0.5% respectively, whereas MBP-Ro60 aa 149–244 and aa 193–236 had a negligible effect on β_2GPI binding (6.1±0.3% and 6.5±3.4% inhibition respectively). These data support Ro60 aa 82–146 as being the predominant β_2GPI binding region. The 2-fold greater inhibition observed with MBP-Ro60 aa 82–192 compared to the truncated MBP-Ro60 aa 82–146 suggests that β_2GPI may favor the conformation presented by the larger Ro60 fragment.

In a previous study Ro60 was shown to bind β_2GPI via its fifth domain (aa 244–326), (6) a structurally distinct part of the molecule defined by a lysine-rich patch and hydrophobic loop. To predict potential Ro60 binding sites within domain V of β_2GPI, hypothetical docking experiments using a previously reported homology model of human Ro60 (22) and human β_2GPI (8) were performed using Swiss PdbViewer. Kyte-Doolittle plots reflected a high degree of charge complementarity in the primary sequences of Ro60 aa 82–146 and β_2GPI domain V. More specifically, manual docking taking into account both conformation complementarity and electrostatics indicated a potential surface interaction between the relatively acidic cleft formed by the mapped Ro60 aa 82–146 and a basic region adjacent to the β_2GPI domain V hydrophobic loop (Figure 3B). Interestingly, the Ro60 binding site on

β_2GPI was within the plasmin cleavage site, Lys317-Thr318. This suggests that proteolytic cleavage of β_2GPI by plasmin may abrogate binding to Ro60 and promote the formation of pathogenic anti-Ro60-apoptotic cardiomyocyte complexes. Precedent for this possibility is that proteolytic cleavage of β_2GPI by plasmin prevents binding to phospholipids(11).

Plasmin cleavage of β_2GPI prevents binding to apoptotic cardiomyocytes and promotes anti-Ro60 binding

To determine whether plasmin cleavage of β_2GPI results in a loss of specificity for Ro60 on apoptotic cardiomyocytes, a plasmin-cleaved form of β_2GPI was prepared (19) and binding to Ro60 assessed. Recombinant MBP-Ro60 bound to immobilized native β_2GPI by ELISA (OD 1.0±0.02 compared to MBP control 0.1±0.01) but did not bind to the plasmin cleaved β_2GPI (OD 0.1±0.02). Flow cytometry experiments confirmed that plasmin cleavage of β_2GPI resulted in reduced binding to Ro60 on the apoptotic cardiomyocytes (Figure 3C). To determine the effect of plasmin-cleaved β_2GPI on the opsonisation of apoptotic cells by anti-Ro60 antibody, maternal anti-Ro60 IgG was co-incubated with either native or cleaved β_2GPI and added to late apoptotic cardiomyocytes. To assess whether plasmin reverses the protective effect of β_2GPI, anti-Ro60 IgG and native β_2GPI in the presence or absence of plasmin were incubated together with apoptotic cells. As expected, the opsonization of apoptotic cardiomyocytes by anti-Ro60 IgG (MFI 3,445±102) was significantly inhibited by β_2GPI (MFI 436±90) ($p=0.006$). The addition of plasmin to this system reversed the protective effect of β_2GPI and promoted anti-Ro60 IgG opsonization at levels similar to those achieved in the absence of β_2GPI (MFI 3,378±66) (Figure 3D).

Plasmin cleavage of β_2GPI could account for the reduced binding of umbilical cord β_2GPI in the quantitative ELISA (Figure 2A). However, plasmin-cleaved β_2GPI could not be directly measured by ELISA (due to unavailability of monospecific reagent). Therefore, plasmin cleaved β_2GPI was compared to native purified β_2GPI on ELISA. Native β_2GPI gave a stronger signal on ELISA compared to cleaved β_2GPI (range of reduction in binding 19–51%, $p=0.0001$). The reduction in binding was presumably due to the loss of an epitope which was recognised by the coating anti-β_2GPI polyclonal antibody since immobilized plasmin-cleaved β_2GPI showed similar reactivity with an anti-β_2GPI domain I monoclonal antibody as native β_2GPI (OD 1.57±0.14 vs. 1.54±0.14, n=3). This discrepancy in binding between native and cleaved β_2GPI led to the speculation that there might be increased cleavage of β_2GPI in fetuses with cardiac NL. To determine whether lower levels of β_2GPI in umbilical cord blood from cardiac NL cases was due to a reduced concentration of β_2GPI or increased cleavage of β_2GPI by plasmin, immunoblots were repeated using the same panel of 9 umbilical cord bloods (used in Figure 2A) but probed with the anti-β_2GPI domain I monoclonal antibody (which detects both intact and cleaved β_2GPI equally). Membranes probed with this monoclonal antibody still revealed lower levels of β_2GPI in the umbilical cord bloods from the 5 neonates with cardiac NL compared to the 4 non-cardiac NL neonates equivalent to the results obtained when the membranes were probed with the polyclonal antibody (Figure 2A). This finding suggests that the lower levels of β_2GPI observed by ELISA and immunoblot are due to a lower concentration of circulating β_2GPI protein rather than increased levels of plasmin-cleaved β_2GPI in neonates with cardiac NL.

Discussion

In the present study, we show that β_2GPI competes with maternal anti-Ro60 IgG for binding to the surface of human fetal cardiomyocytes rendered apoptotic. A reasonable extension of these data to the pathogenesis of cardiac NL is the prediction that fetal levels of circulating β_2GPI represent a protective factor which averts tissue injury by displacing antibody binding. The displacement by β_2GPI may explain, in part, the absence of anti-Ro60 IgG

binding to apoptotic cells in vivo in a murine transplacental model, (28) or passive transfer xenograft model (29).

The hypothesis of β_2GPI as a protective factor in cardiac NL is supported by our finding that levels of circulating β_2GPI are lower in neonates with cardiac NL compared to unaffected neonates. Several factors could account for the lower levels of β_2GPI observed in the neonates with cardiac NL. Previous studies of cardiac tissue from fetuses dying with cardiac NL demonstrated increased apoptotic cells in the septal region (5). Therefore, decreased levels of β_2GPI might be secondary to absorption from the circulation by binding to the apoptotic cells. However, this possibility is unlikely for several reasons a) apoptosis occurs earlier in development and not necessarily at term b) the binding of β_2GPI should be displaced by anti-Ro60 antibodies (if equivalent as in the case of twins) in both affected and unaffected fetuses. Alternatively, lower levels of β_2GPI may be a primary factor in antibody mediated injury due to a genetic predisposition and not a secondary effect. It is known that plasma levels of β_2GPI vary among individuals (ranging from undetectable to 350 μg/ml in adults) which may be under genetic control (30). For example, substitution of C with A at the β_2GPI transcriptional initiation site is a causative mutation that affects gene expression at the transcriptional level and ultimately β_2GPI plasma concentrations (31). Although it has been reported that African Americans have lower levels than Caucasians due to a genetic polymorphisms, the lowest levels detected were not present in the few African American patients (data not shown). The disparate levels in the discordant monozygotic twins suggest that genetics do not fully account for reduced β_2GPI. Finally, maternal anti-β_2GPI antibodies were not associated with a loss of the protective function of β_2GPI or the low levels of β_2GPI observed in the umbilical cord bloods.

Molecular studies revealed the β_2GPI binding site on Ro60 to be within amino acids 82–146. In a recent report, we showed that this region of Ro60 also contains an apotope which is recognised by 45% of patients with SLE and isolated anti-Ro60 responses (22). However, β_2GPI inhibited the opsonisation of apoptotic cells by IgG from patients with anti-Ro60 autoantibodies, not just those with reactivity to the aa 82–146 apotope (data not shown). This suggests that β_2GPI inhibits the binding of anti-Ro60 IgG by steric hindrance, where upon binding to Ro60 aa 82–146, β_2GPI undergoes a conformational change and masks other Ro60 apotopes. Interestingly, Ro60 binds to β_2GPI within the plasmin cleavage site, residues 317–318, of domain V. Based on these findings, it is likely that a neonate with less circulating β_2GPI would be more vulnerable to the effects of plasmin cleavage than a neonate with higher levels of β_2GPI. These findings are in accord with those of Briasouli and colleagues (32) who demonstrated that binding of anti-Ro60 antibodies to apoptotic cardiomyocytes results in an increased and altered distribution of uPAR as well as enhanced activation of the urokinase-type plasminogen activator protease (uPA). In binding experiments using FACS analysis, these authors confirmed that preincubation of apoptotic cardiomyocytes with β_2GPI markedly decreased the binding of IgG from a mother whose child had cardiac NL to the apoptotic cardiomyocytes. Consistent with the notion that the binding of anti-Ro60 contributes to a pathway leading to an enhanced activation of the urokinase-type plasminogen activator protease, β_2GPI added prior to the cardiac NL-IgG attenuated plasminogen activation.

While the clinical association between maternal anti-Ro60 autoantibodies and cardiac NL is strong (33), the rarity and unique fetal vulnerability suggest that other variables are required for disease expression. Both the levels of circulating β_2GPI and extent of plasmin cleavage may be highly permissive with regard to the balance of anti-Ro60 antibody binding. Either intrinsically lower levels based on genetic predisposition or cleavage generated by plasminogen activation would confer loss of protection in fetuses exposed to maternal anti-Ro60 antibodies. Since Ro60 is the first of the Ro/La autoantigens to be exposed on the

surface of cells undergoing apoptosis (17), it is plausible that preventing the formation of pathogenic anti-Ro60-apoptotic cell complexes with β_2GPI may facilitate clearance and protect the fetal heart from antibody-mediated tissue injury.

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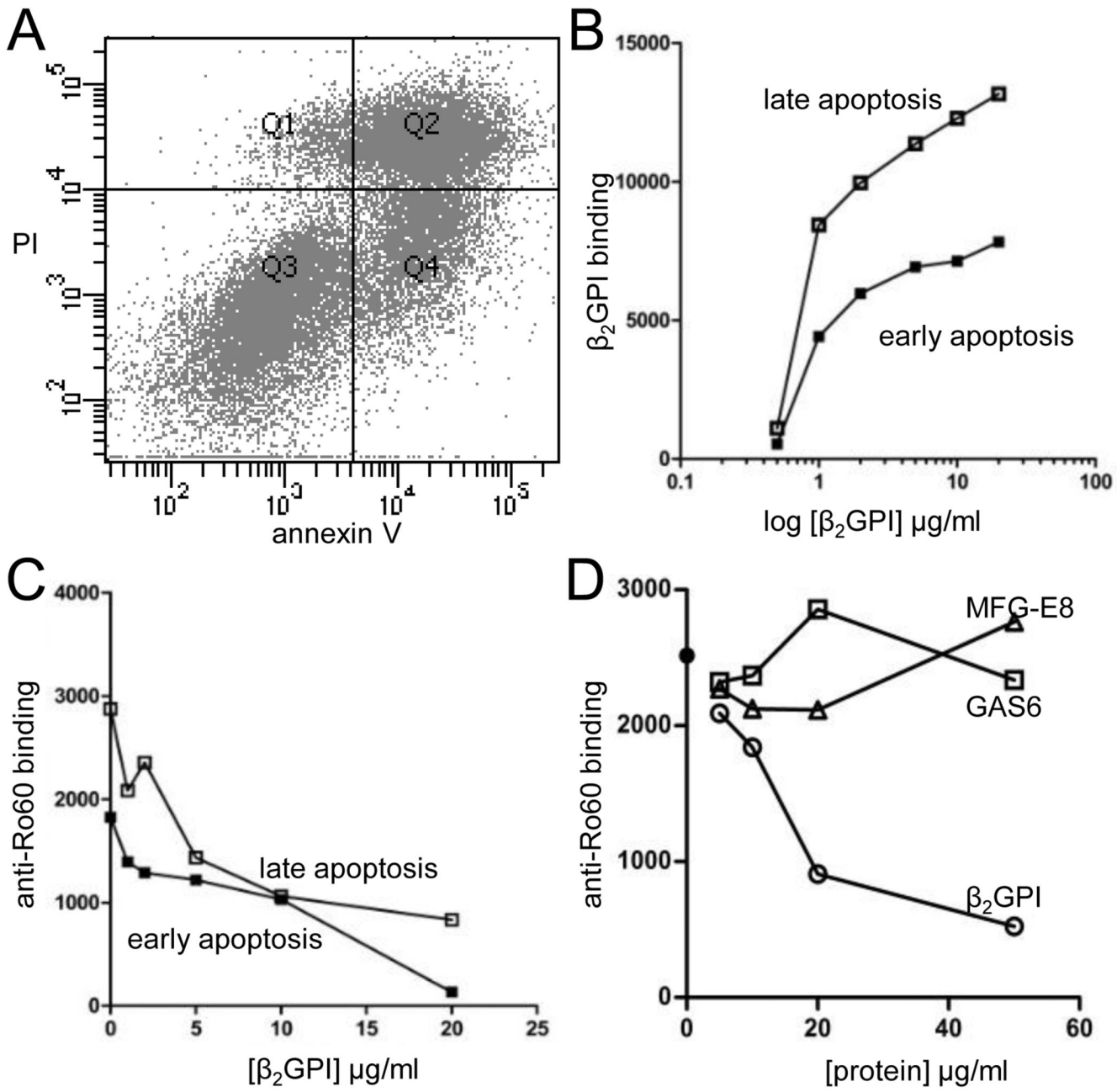

FIGURE 1.

Purified native human β_2GPI binds to apoptotic human fetal cardiomyocytes and inhibits binding of maternal anti-Ro60 IgG. *(A)* Apoptotic cardiomyocyte populations were gated based on Annexin V and propidium iodide (PI) staining. Annexin V-positive, PI-negative cells (Quadrant, Q4) were termed early apoptotic and Annexin V/PI-positive cells (Q2) were late apoptotic. *(B)* β_2GPI bound both early and late populations in a dose-dependent and saturable manner. (C) Increasing concentrations of β_2GPI inhibited the binding of anti-Ro60 IgG to both early and apoptotic cardiomyocyte populations. (D) The inhibition of anti-Ro60 IgG binding to apoptotic cardiomyocytes is specific for β_2GPI as GAS6 and MFG-E8 do not alter antibody binding.

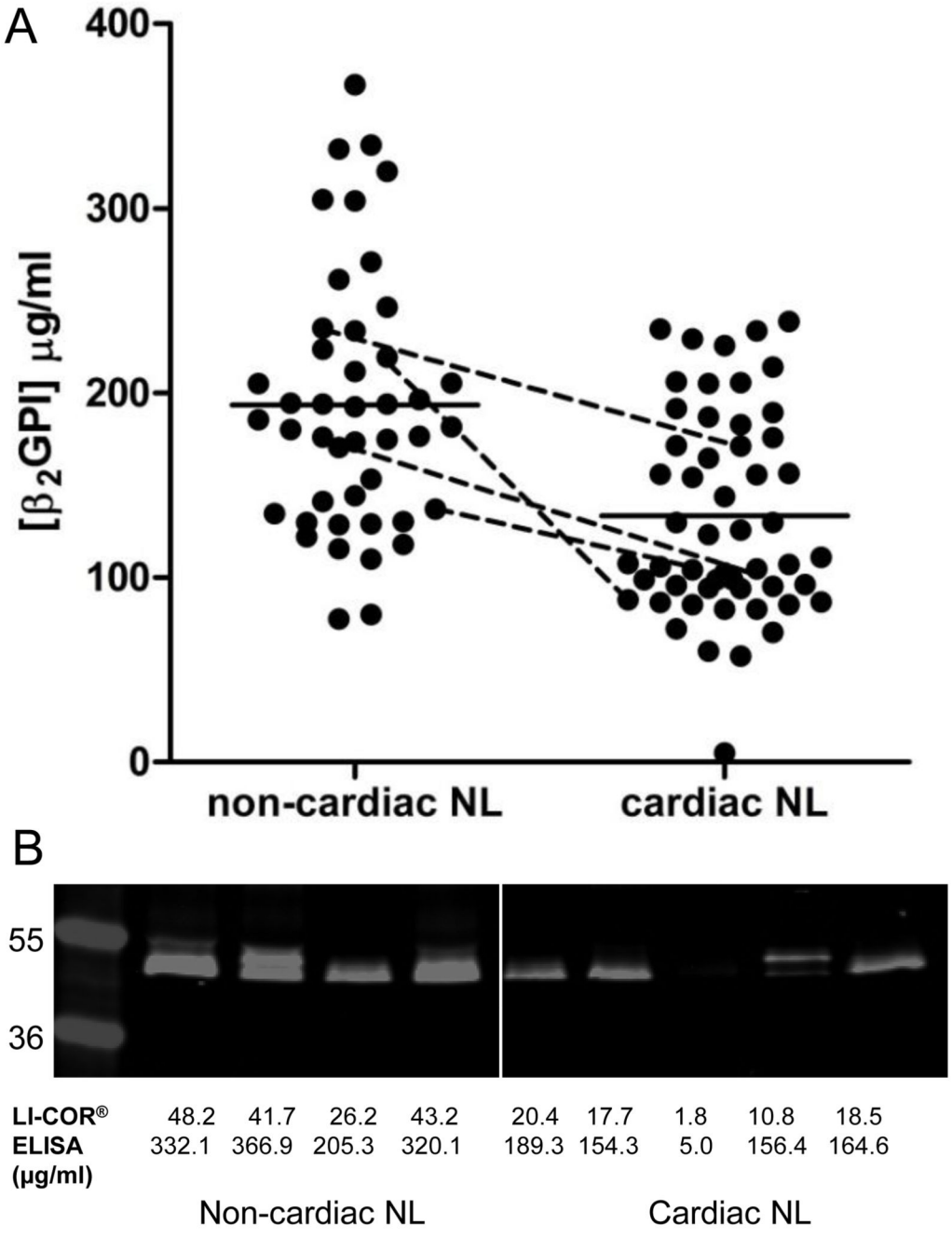

FIGURE 2.

β_2GPI levels are lower in anti-Ro60 exposed neonates with cardiac NL compared to non-cardiac NL. *(A)* β_2GPI concentration, measured by ELISA, in umbilical cord blood from children affected by cardiac NL (n=53) and non-cardiac NL controls (n=44). Solid horizontal lines represent mean β_2GPI concentration. Dashed lines connect β_2GPI levels in umbilical cord blood from twins discordant for cardiac NL. *(B)* Immunoblot analysis of a panel of 9 umbilical cord blood samples (4 non-cardiac NL and 5 cardiac NL) probed with anti-β_2GPI antibody were quantitatively consistent with those measured by ELISA.

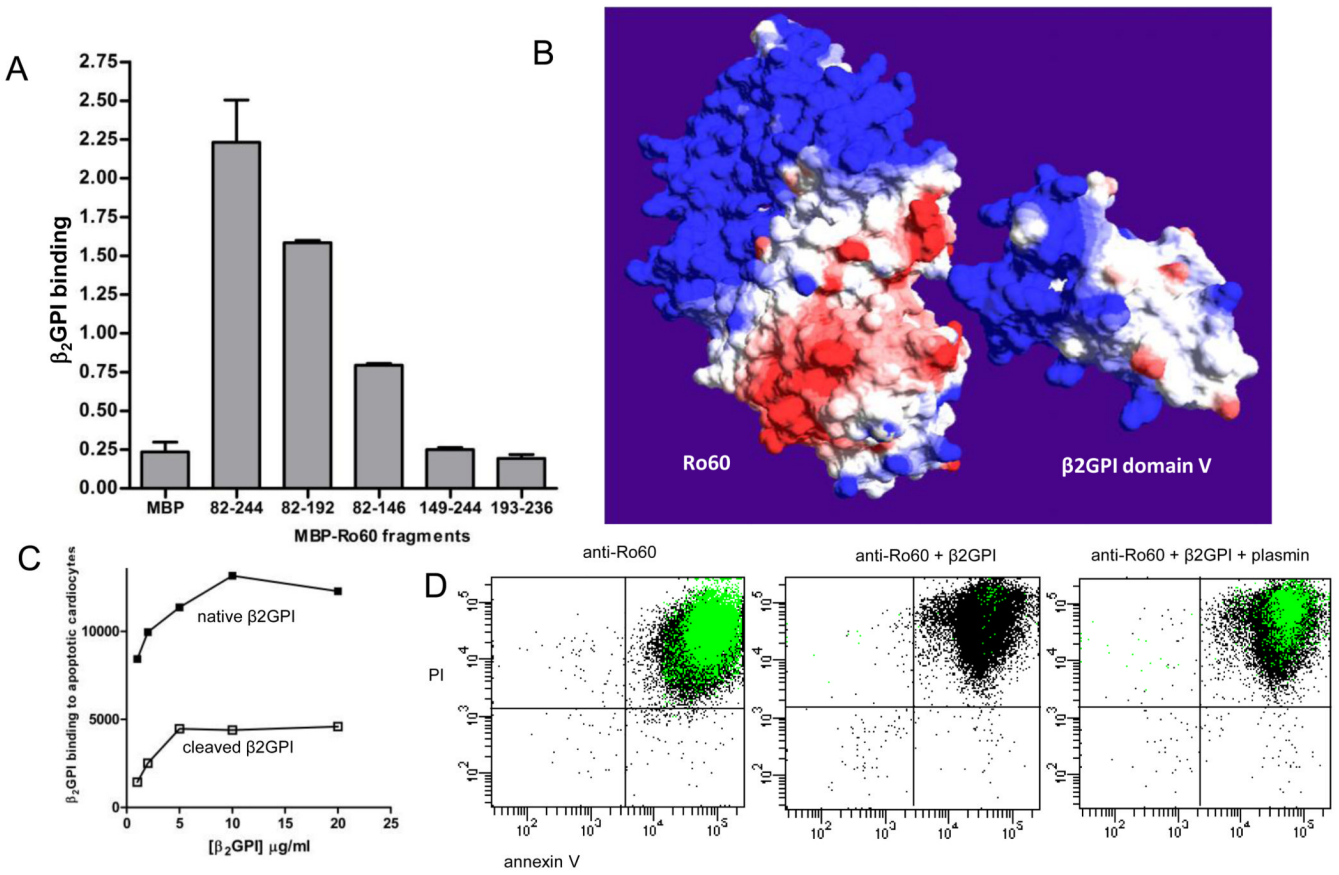

FIGURE 3.

The Ro60 binding site on β_2GPI is within the plasmin cleavage site, Lys317-Thr318. *(A)* The β_2GPI binding site on Ro60 was measured by ELISA using recombinant maltose binding protein (MBP) expressing various regions of Ro60. Ro60 fragments encompassing amino acids (aa) 82–244, aa 82–192, and aa 82–146 bound to purified native human β_2GPI compared to MBP-Ro60 aa 149–244, aa 193–236, and MBP control. Values are the mean ± standard deviation (SD) of triplicate determinations (n=3). *(B)* Molecular surface models of Ro60 and β_2GPI with the β_2GPI fifth domain oriented towards a cleft in Ro60 formed by the mapped aa 82–146 region. Surface colours are calculated using a coulombic algorithm for electrostatic potential where blue is positive charge, white is neutral, and red is negatively charged. *(C)* The binding of β_2GPI to apoptotic cardiomyocytes is abrogated by plasmin cleavage. *(D)* Plasmin reverses the β_2GPI-mediated inhibition of anti-Ro60 binding to apoptotic cells. Representative flow cytometry dot plots depicting the binding of anti-Ro60 IgG (green) to late apoptotic cells (n=5).

Table I

Clinical and demographic characteristics of anti-Ro60 exposed neonates with cardiac NL compared to those without cardiac NL

	Cardiac NL* (N=53)	Non-Cardiac NL** (N=44)	p Value
Sex of Child			0.22
Male	24 (45%)	25 (57%)	
Female	29 (55%)	17 (39%)	
NA	0 (0%)	2 (4%)	
Race/ethnicity			0.78
White	43 (81%)	36 (82%)	
Black	3 (6%)	3 (7%)	
Hispanic	1 (2%)	4 (9%)	
Asian	4 (7%)	1 (2%)	
Other/NA	2 (4%)	0 (0%)	
Antibody Status			0.68
Anti-Ro+/La+	29 (55%)	27 (61%)	
Anti-Ro+/La−	24 (45%)	17 (39%)	
Medication			
Dexamethasone	26 (49%)	3 (7%)	0.0001
Delivery			
C-section	37 (70%)	10 (23%)	0.0001
Vaginal	3 (6%)	16 (36%)	
NA	13 (24%)	18 (41%)	
Birth Weight (g)	2682 (±647)+	3124 (±572)++	0.007
Gestational age(weeks)	37 (±2)#	38 (±2)##	0.018

*3rd degree block (n=47), 2nd degree (n=2), cardiomyopathy (n=3), sinus bradycardia (n=1)

**rash (n=5), liver complications (n=1)

+n=36,

++n=29,

#n=44,

##n=31

Birth weight and gestational age are presented as mean±SD. All other data are reported as N (%). NL, neonatal lupus.