Concise report

A central role of plasmin in cardiac injury initiated by fetal exposure to maternal anti-Ro autoantibodies

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Abstract

Objective. Cardiac neonatal lupus (cardiac-NL), initiated by surface binding of anti-Ro60 autoantibodies to apoptotic cardiocytes during development, activates the urokinase plasminogen activator/urokinase plasminogen activator receptor (uPA/uPAR) system. Subsequent accumulation of apoptotic cells and plasmin generation facilitates increased binding of anti-Ro60 by disrupting and cleaving circulating β2-glycoprotein I ([β2GPI]) thereby eliminating its protective effect. The association of soluble levels of components of the uPA/uPAR system with cardiac-NL was examined.

Methods. Levels of the uPA/uPAR system were assessed by ELISA in cord blood and immunohistological evaluation of autopsies.

Results. uPA, uPAR and plasminogen levels were each significantly higher in cord blood from cardiac-NL (n = 35) compared with non-cardiac-NL (n = 26) anti-Ro-exposed neonates: 3.3 ± 0.1 vs 1.9 ± 0.05 ng/ml (P < 0.0001), 6.6 ± 0.3 vs 2.1 ± 0.2 ng/ml (P < 0.0001) and 435 ± 34 vs 220 ± 19 ng/ml (P < 0.0001), respectively. In three twin pairs discordant for cardiac-NL, the twin with cardiac-NL had higher levels of uPA, uPAR and plasminogen than the unaffected twin (3.1 ± 0.1 vs 1.9 ± 0.05 ng/ml; P = 0.0086, 6.2 ± 1.4 vs 2.2 ± 0.7 ng/ml; P = 0.147 and 412 ± 61 vs 260 ± 27 ng/ml; P = 0.152, respectively). Immunohistological evaluation of three hearts from fetuses dying with cardiac-NL revealed macrophages and giant cells expressing uPA and plasminogen in the septal region.

Conclusion. Increased soluble uPA, uPAR and plasminogen in cord blood and expression in affected tissue of fetuses with cardiac-NL supports the hypothesis that fetal cardiac injury is in part mediated by plasmin generation initiated by anti-Ro binding to the apoptotic cardiocyte.

Key words: apoptosis, fibrosis, inflammation.

Introduction

Cardiac neonatal lupus (cardiac-NL) represents a pathological readout of passively acquired autoimmunity that occurs during the second trimester of pregnancy and is almost universally associated with maternal antibodies to SSA/Ro ribonucleoproteins. The mechanism by which maternal antibodies initiate and eventuate cardiac scarring has been challenging to define, in part because the target cardiac antigens are normally sequestered intracellularly. In vitro and in vivo studies suggest that apoptosis may be a key step in facilitating the accessibility of intracellular antigen to extracellular maternal autoantibodies. Previous studies demonstrated that binding of anti-SSA/Ro antibodies to apoptotic fetal cardiocytes impairs their removal by healthy cardiocytes. Immunohistological evaluation supports the in vitro findings since exaggerated apoptosis has been observed in the septal region of several hearts from fetuses dying with cardiac-NL [1, 2].

Recent studies have shown that binding of anti-Ro60 antibodies to the surface of apoptotic cardiocytes leads to increased urokinase plasminogen activator/urokinase plasminogen activator receptor (uPA/uPAR)-dependent plasminogen activation and plasmin generation. Among the downstream consequences of this protease activation...
is the cleavage of β2-glycoprotein I (β2GPI), which, when intact, competes with anti-Ro60 binding to Ro60 on the apoptotic cardiocyte [3]. In addition to the loss of a potential cardioprotective effect, plasmin generation activates latent TGF-β, hypothesized to promote scarring through the excess production of collagen [4].

Dysregulation of the uPA/uPAR system via increased uPA/uPAR-dependent plasminogen activation is expected to result in the generation and accumulation of soluble uPAR, a pathological readout in various diseases and relevant to cardiac-NL [5–8]. High expression of uPAR in a small tissue volume can elevate circulating levels to a measurable extent, be detected systematically and is stable in blood and urine. Accordingly, this study was initiated to evaluate the in vivo significance of the uPA/uPAR system in the pathogenesis of cardiac-NL. Readouts included the measurement of uPA, uPAR and plasminogen levels in affected fetuses and immunohistochemical evaluation in hearts from fetuses dying with cardiac-NL.

**Patients and methods**

**Patients and controls**

Umbilical cord serum or plasma was obtained from neonates of anti-Ro60-positive mothers enrolled in the Research Registry for Neonatal Lupus (RRNL) or the PR Interval and Dexamethasone Evaluation (PRIDE) database. Each database has institutional review board approval for evaluation of de-identified information and informed consent was obtained from these patients in accordance with the Declaration of Helsinki. The RRNL and its informed consent documents were approved by the New York University School of Medicine Institutional Review Board. This covers ethical approval for all studies involving retrospective analysis of autopsy or clinical materials submitted by the families at our request. The New York University School of Medicine Institutional Review Board does not require ethical approval for the use of de-identified information from both the RRNL and PRIDE. A neonate was considered to have cardiac-NL based on (i) the presence of heart block and/or cardiomyopathy, (ii) the presence of antibodies to SSA/Ro in the maternal serum. Neonates categorized as non-cardiac-NL met inclusion (ii) above but had no evidence of any cardiac abnormalities.

**Quantitative measurement of uPA, uPAR, plasminogen and plasmin-anti-plasmin complexes in umbilical cord blood by ELISA**

Umbilical cord serum or plasma was diluted 1/1000 in 1% BSA/PBS. For the determination of uPA and plasminogen levels, commercial ELISA kits were used (American Diagnostica, Stamford, CT, USA) according to the manufacturer’s instructions. For the measurement of uPAR levels, a quantitative ELISA kit was used (R&D, Minneapolis, MN, USA). Levels of β2-glycoprotein I in these samples have been previously reported [3].

**Immunohistochemistry**

Hearts were examined from three fetuses dying with cardiac-NL (at 40 weeks as described previously [9], 21 weeks and 22 weeks), one anti-Ro-exposed neonate with no cardiac disease dying at 24 weeks after premature delivery for maternal HELLP syndrome and one otherwise healthy non-Ro-exposed fetus electively terminated at 24 weeks. The Triangle of Koch was dissected from the hearts to obtain the AV nodal area and was used to generate formalin-fixed paraffin-embedded blocks. However, in the 21-week cardiac-NL fetus, only the base of the heart was available for analysis and conducting tissues from this area were obtained. Five-micron sections were cut and placed on plus slides. These were immersed in Trilogy rinse (Cell Marque, Hot Spring, AR, USA) and placed in an electric pressure cooker until reaching 127°C and 23 psi. Endogenous peroxidase activity was blocked for 5 min in 3% H2O2 in PBS. Slides were incubated with either rabbit anti-plasminogen (Sigma-Aldrich, St Louis, MO, USA), rabbit anti-uPA (American Diagnostica, Stamford, CT, USA), goat anti-uPAR (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or isotype control (Alpha Diagnostic International, San Antonio, TX, USA) for 30 min at room temperature followed by a rabbit or goat HRP polymer conjugate (SuperPicTure, Invitrogen, Camarillo, CA, USA) for 10 min. Finally, slides were stained with Impact DAB (Vector Labs, Burlingame, CA, USA) for 3 min and counterstained with haematoxylin (Richard-Allen, Kalamazoo, MI, USA).

**Statistical analysis**

Dichotomous and continuous variables between cardiac-NL and non-cardiac-NL neonates were compared by Fisher’s exact test and Mann-Whitney test, respectively. Levels of uPA, uPAR and plasminogen in umbilical cord blood from twins were compared by paired t-test. The levels of uPA, uPAR and plasminogen were compared with β2GPI titre by linear regression. Two-sided P-values <0.05 were considered statistically significant.

**Results**

**Increased uPA, uPAR and plasminogen levels in umbilical cord blood of fetuses with cardiac-NL**

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 uPA, uPAR and plasminogen levels in the pathogenesis of disease. Umbilical cord blood from 61 anti-Ro60-exposed infants was studied, 35 with cardiac-NL and 26 with no cardiac manifestations (Table 1). Of those affected, 32 had congenital heart block (31, third degree; 1, second degree), 1 had first degree block and evidence of cardiomyopathy, 2 had an isolated cardiomyopathy and 1 had sustained sinus bradycardia. Of the non-cardiac-NL neonates (many with affected siblings), 24 were completely healthy and 2 had cutaneous NL.
Birth weight and gestational age are presented as mean ± S.D. uPA, uPAR and plasmin levels are presented as median ± S.E.M. All other data are reported as n (%).

**Table 1** Clinical and demographic characteristics and uPA, uPAR and plasmin levels of anti-Ro60-exposed neonates with cardiac NL compared with those without cardiac NL.

<table>
<thead>
<tr>
<th></th>
<th>Cardiac NL (^a) (n = 35)</th>
<th>Non-cardiac NL (^b) (n = 26)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex of child</td>
<td></td>
<td></td>
<td>0.30</td>
</tr>
<tr>
<td>Male</td>
<td>16 (46)</td>
<td>16 (62)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>19 (54)</td>
<td>10 (38)</td>
<td></td>
</tr>
<tr>
<td>Race/ethnicity</td>
<td></td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>White</td>
<td>28 (80)</td>
<td>21 (81)</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>2 (6)</td>
<td>2 (8)</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>1 (3)</td>
<td>3 (11)</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>4 (11)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Antibody status</td>
<td></td>
<td></td>
<td>0.61</td>
</tr>
<tr>
<td>Anti-Ro+/La+</td>
<td>19 (54)</td>
<td>12 (46)</td>
<td></td>
</tr>
<tr>
<td>Anti-Ro+/La−</td>
<td>16 (46)</td>
<td>14 (54)</td>
<td></td>
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<tr>
<td>Medication</td>
<td></td>
<td></td>
<td>0.0012</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>16 (46)</td>
<td>1 (4)</td>
<td></td>
</tr>
<tr>
<td>Delivery</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>C-section</td>
<td>25 (71)</td>
<td>4 (15)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Vaginal</td>
<td>2 (6)</td>
<td>9 (25)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>8 (23)</td>
<td>13 (50)</td>
<td></td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>2578 (±616)(^c)</td>
<td>3190 (±511)(^d)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>36 (±2)(^e)</td>
<td>38 (±2)(^f)</td>
<td>0.0027</td>
</tr>
</tbody>
</table>

Birth weight and gestational age are presented as mean ± S.D. uPA, uPAR and plasmin levels are presented as median ± S.E.M. All other data are reported as n (%). \(^a\)Third-degree block (n = 30), second-degree block (n = 1), cardiomyopathy (n = 3), sinus bradycardia (n = 1); \(^b\)rash (n = 2); \(^c\)n = 26; \(^d\)n = 30; \(^e\)n = 15.

The demographic characteristics, mother's antibody status and medication, method of delivery, infant birth weight and gestational age are shown in Table 1. As expected, mothers of children with cardiac-NL were more likely to have taken dexamethasone and be delivered by Caesarean section. Cardiac-NL children were more frequently born prematurely and of lower birth weight than the non-cardiac-NL controls.

uPA, uPAR and plasminogen levels were significantly higher in cardiac-NL compared with non-cardiac-NL children (3.3 ± 0.1 vs 1.9 ± 0.05 ng/ml, P < 0.0001; 6.6 ± 0.3 vs 2.1 ± 0.2 ng/ml, P < 0.0001 and 435 ± 34 vs 220 ± 19 ng/ml, P < 0.0001, respectively) (Fig. 1P−R). In three twin pairs discordant for cardiac-NL, the twin with cardiac-NL had higher levels of uPA (3.1 ± 0.1 vs 1.9 ± 0.05 ng/ml; P = 0.0086) compared with the non-cardiac-NL twin. Levels of uPAR and plasminogen were also higher in the cardiac-NL twin but the differences did not reach significance (6.2 ± 1.4 vs 2.2 ± 0.7 ng/ml; P = 0.147 and 412 ± 61 vs 260 ± 27 ng/ml; P = 0.152, respectively) (Fig. 1P−R). There was no association between levels of uPA, uPAR and plasminogen and gender, dexamethasone use, delivery, birth weight or gestational age of the fetuses. Supporting a cardioprotective role, the levels of circulating β2GPI were inversely correlated with the levels of uPAR, uPA and plasminogen when the groups were combined (P < 0.0001, P = 0.0001 and P = 0.0023, respectively), but for each group there was only a trend (data not shown).

uPA and plasminogen are detected in the hearts of three fetuses dying with cardiac-NL

In vivo evidence of uPA/uPAR and plasminogen activity in cardiac-NL hearts was sought. In the three hearts from fetuses dying with cardiac-NL, the histological features included the presence of calcified myocytes, macrophages, giant cells, fibroblasts and the loss of normal AV nodal cardiocytes. In the 21-week heart, there was also an extensive lymphocytic infiltrate at the edges of the injured conducting tissues that extended into adjacent normal myocardium. Plasminogen expression was prominent in giant cells and inflammatory cells infiltrating the conducting tissue (Fig. 1A–C). In unaffected hearts, plasminogen was only present along the endothelium of normal arteries (Fig. 1D and E). uPA was also expressed in inflammatory cells infiltrating the conducting tissues in the cardiac-NL hearts, but absent in the non-cardiac-NL hearts (Fig. 1F–J). uPAR staining was not observed in any of the hearts (Fig. 1L–N). An isotype control antibody (normal rabbit IgG) was negative in all of the tissues (Fig. 1O).

**Discussion**

Binding of anti-Ro60 antibody to apoptotic cardiocytes leads to increased uPAR and subsequent elevated uPA dependent plasminogen activation. Accordingly, we hypothesized that the cycle of amplification of anti-Ro60 binding/uPAR increase/plasminogen activation/uPAR cleavage would result in the accumulation of uPA in the cord blood and tissue. In support of this, levels of uPA, uPAR and plasminogen in the umbilical cord blood from anti-Ro-exposed neonates with cardiac-NL were significantly elevated compared with samples obtained from anti-Ro-exposed neonates without any cardiac disease. Histological evidence of increased expression of uPA and plasminogen on the infiltrating macrophages and giant cells in the autopsy specimens from fetuses dying with cardiac-NL further supports the in vitro data.

While it remains unclear why all anti-Ro60-exposed fetuses do not generate increased uPA/uPAR activity, β2GPI may be a factor, by conferring a break on the uPA/uPAR activation cycle by competing with anti-Ro60 for initial binding to the apoptotic cardiocyte. The increased uPA/uPAR-dependent plasmin would generate cleaved β2GPI, which does not bind to Ro60, amplifying the binding of antibodies to the apoptotic cardiocytes and exacerbating injury. These considerations are consistent with the finding that levels of β2GPI inversely correlated with levels of uPA, uPAR and plasminogen in neonates with cardiac-NL.

Multiple forms of soluble uPAR and uPA exist, attributed to domain cleavage and alternative splicing. uPAR consists of three homologous domains, I, II and III. In addition to the activation of plasminogen, both uPA and plasminogen are capable of cleaving uPAR between domain I and II,
Conducting system tissue from three cardiac-NL hearts (A–C) showing plasminogen staining of macrophages and giant cells (black arrows). (D) AV node from anti-Ro-exposed heart and (E) control heart showing weak endothelial staining. Conducting system tissue from three cardiac-NL hearts (F–H) showing uPA staining in inflammatory and endothelial cells. (I) uPA endothelial staining in the AV nodal region of anti-Ro-exposed heart and (J) control heart. (K) Colon cancer showing rare staining for uPAR. (L–M) AV node from two cardiac-NL hearts or (N) anti-Ro-exposed heart showing no uPAR staining. (O) An IgG-negative control stain from the non-cardiac-NL heart (original magnification A–J ×160, K–O ×100). uPA (P), uPAR (Q) and plasminogen (R) concentration, measured by ELISA, in umbilical cord blood from children affected by cardiac-NL (n = 35) and from non-cardiac-NL controls (n = 26). Solid horizontal lines represent median concentration. Dashed lines connect levels in cord blood from twins discordant for cardiac-NL.
releasing domain I, uPAR(I) and leaving the cleaved form, uPAR(II-III), on the cell surface. The cleavage of uPAR exposes a chemotactic epitope on the cell surface that in a soluble form [uPAR (II-III)] also acts as a potent chemoattractant for monocytes by activating FPR1 [8]. uPA itself is a chemokine contributing to the infiltration of relevant immune cells [10]. Relevant to cardiac-NL, cleaved uPA and uPAR fragments may contribute to the macrophage infiltration observed in autopsies described herein and in previous reports [11, 12].

Immunohistological evaluation of the cardiac-NL hearts showed strong uPA and plasminogen expression on infiltrating macrophages and giant cells. Macrophage expression of uPA may be induced by uPA/uPAR-dependent activation of TGF-β signaling following binding of the anti-Ro antibodies to the apoptotic cardiocytes [4, 13]. Plasmin at sites of inflammation triggers cytokine expression [14, 15] and activation of MMPs including MMP9, which have been reported to facilitate macrophage fusion and generation of giant cells [16]. uPA expression on the macrophages has been associated with a profibrotic phenotype, thus plasminogen activation appears to be a critical link between the accumulation of macrophages and collagen production in the heart in response to injury [11]. Of relevance to cardiac-NL, targeted overexpression of uPA or deficiency of PAI-1 in macrophages has been shown to specifically drive cardiac fibrosis in mice [12, 17, 18].

Although uPAR levels were elevated in cord blood, there was no expression in the immunohistological studies. This could be attributed to the postmortem interval or the occupancy of uPAR by uPA, with masking of critical epitopes inhibiting recognition by a uPAR-specific antibody. This phenomenon has been described in in vitro studies [19].

Collectively these data support our in vitro studies that deregulation of the uPA/uPAR system is a marker of cardiac-NL and indicative of its causative role in disease progression. Measurement of soluble levels of uPA/uPAR components may have future prognostic value and these data reinforce that therapy targeted to this system might represent a novel approach.

### Funding
This work was supported by National Institutes of Health grants 1K01AR060302 (to P.B.), R01 AR42455-16 and N01-AR-4-2271 (to J.P.B. and R.M.C.).

### Disclosure statement
The authors have declared no conflicts of interest.

### References


### Rheumatology key message
- Increased expression and levels of soluble uPA, uPAR and plasminogen where found in cord blood in affected tissue of fetuses with cardiac-NL.


