Upregulated Serum Granulysin Levels in Women With Antiphospholipid Syndrome-Associated Recurrent Miscarriages Are Downregulated by Heparin Treatment

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Abstract

Background

Antiphospholipid antibody syndrome is the major cause of recurrent pregnancy loss (RPL) and associated with inflammation. Granulysin is a cytotoxic protein secreted by cytotoxic T cells, natural killer cells, and natural killer T cells that is present in abundance in the decidua. It activates innate and cellular immunity simultaneously, and also induces miscarriage. As a treatment, heparin is widely used for the patients with RPL, and exhibits the antithrombotic and anti-inflammatory activities, and angiogenesis.

Methods

We hypothesized that granulysin is an important factor in inducing miscarriage. Here, we evaluated the changes of serum granulysin level before and 1 week after the commencement of heparin treatment for the patients with RPL.

Results

The serum granulysin levels before heparin treatment were significantly higher in women who tested positive for one or more types of antiphospholipid antibody (2.75 ± 1.03 vs. 2.44 ± 0.69; $P = 0.0341$ by Welch's *t*-test), particularly anti-phosphatidylethanolamine antibodies (IgG: 2.98 ± 1.09 vs. 2.51 ± 0.86; $P = 0.0013$, IgM: 2.85 ± 1.09 vs. 2.47 ± 0.77; $P = 0.0024$ by Welch's *t*-test). After heparin treatment for 1 week, the serum granulysin levels were reduced significantly ($P = 0.0017$ by paired *t*-test). The miscarriage rate was significantly higher in women whose serum granulysin levels were not reduced by heparin treatment ($P = 0.0086$ by Fisher's exact probability test).

Conclusions

These results suggest that heparin may reduce the incidence of miscarriages by suppressing the serum granulysin levels.

Background

Recurrent pregnancy loss (RPL) is a heterogeneous condition with several etiological factors, such as uterine anomalies, prothrombotic disorders, chromosomal anomalies, endocrine dysfunction, and autoimmune and alloimmune disorders. Among these, antiphospholipid antibody syndrome (APS) is now recognized as one of the most important causes of RPL. An estimated 20–40% of women with RPL test positive for antiphospholipid antibodies (aPLs), compared with 2% of women with normal obstetric histories [1].

APS is primarily characterized by arterial or venous thrombosis, RPL, fetal growth restriction, and preeclampsia in patients positive for aPLs [2]. Observations of extensive infarction and thrombosis in the placentas of aPL-positive women and evidence of systemic thrombosis in patients with APS led to the hypothesis that aPLs cause obstetric complications by inducing thrombosis in the placenta and decidual circulation [3]. However, not all affected placentas display signs of thrombosis or infarction, and most miscarriages occur before placental formation, indicating that other mechanisms are responsible for these obstetric complications.

Recent clinical and experimental studies suggest that the pathophysiology of APS-related pregnancy loss may involve inflammation at the maternal-fetal interface and disruption of normal trophoblast function. Girardi et al. suggested that activation of the complement cascade is necessary for aPL-mediated fetal loss [4]. In addition, the presence of neutrophil extracellular traps (NETs) is crucial in APS. In patients with APS, the neutrophils easily secrete NETs [5], and the thrombi formed include those derived from NETs [6]. It has been postulated that a proportion of RPL cases is attributed to immune reactions [7]. However, the role of immunity in APS pathogenesis remains unclear.

Heparin in combination with aspirin is currently the standard treatment for pregnant women with APS. The rationale underlying this choice of treatment is based on the anticoagulant properties of heparin, since it was originally thought that pregnancy failure associated with aPLs is caused by thrombotic events in the placenta. However, as described above, there may be other mechanisms underlying pregnancy loss, and heparin may be used as an alternative preventive agent. Indeed, heparin has several properties besides its anticoagulant property, including inhibition of aPL binding to β2 glycoprotein I (β2GPI) [8]. Girardi et al. demonstrated that the effectiveness of heparin may be attributed to its complementary inhibitory effects rather than its anticoagulant activity [9]. These observations lead us to hypothesize that changes in immunity may contribute to the pathogenesis and clinical manifestations of APS, and that heparin treatment may modulate immunity in pregnant women with APS.

We used granulysin, a cytolytic granule protein produced by natural killer (NK) cells [10], cytotoxic T lymphocytes (CTLs), natural killer T (NKT) cells, and γδ T cells [11], as an indicator of immunity. Granulysin is a demonstrated marker of cell-mediated immunity [12] as well as perforin and granzyme activities [13]. It is a saposin-like lipid-binding protein [14] that is inserted into cell membranes to induce ion fluxes and apoptosis. Granulysin is composed of 9-kDa and 15-kDa subunits. The 9-kDa subunit exerts cytotoxic effects, whereas the 15-kDa subunit acts as an alarmin [15], which stimulates antigen-presenting cells (APCs) through pattern recognition receptors (PRRs) and is involved in the activation of innate immune cells, including dendritic cells (DCs) [16]. It acts as a damage-associated molecular pattern and induces pre-inflammatory reactions [17]. The 15-kDa subunit promotes APC maturation [18, 19] and immune cell migration [20]. Therefore, granulysin regulates the dual control of the innate and adaptive arms of the immune system.

Granulysin has tumoricidal and antiviral properties and inhibits the growth of pathogenic bacteria, fungi, and parasites *in vitro* [10, 21]. It is also a useful biomarker for transplantation. In acute graft-versus-host

disease, serum granulysin levels are markedly increased and correlate with disease severity [22]. Granulysin has been detected in the endometrium [4] and decidua in early pregnancy [23], and plasma granulysin concentrations are associated with preeclampsia [24]. Recently, Nakashima et al. demonstrated that granulysin produced by uterine NK cells induces apoptosis in extravillous trophoblasts during spontaneous abortion [25]. Tamara et al. also reported that granulysin causes apoptosis and induces miscarriage [26]. Here, we investigated the relationship between APS and immunity by examining serum granulysin levels.

Materials

Patients

Women were recruited from the recurrent miscarriage clinic of the Department of Obstetrics and Gynecology at Nippon Medical School Hospital (Tokyo, Japan). A total of 142 women with a history of recurrent miscarriage (two or more consecutive miscarriages) were included in this study. They were tested for the presence of aPLs, and 32 women whose aPL tests were positive on two or more occasions at least 12 weeks apart were treated with heparin and aspirin. Thirty women whose aPL titers were marginal or only positive on a single occasion were included in this study as the control group, and received aspirin alone.

Laboratory assays for aPL detection

The presence or absence of lupus anticoagulant was determined by measuring the activated partial thromboplastin time and diluted Russell viper venom time, and confirmed using mixing studies and platelet neutralization tests [27, 28]. Standard enzyme-linked immunosorbent assay (ELISA) against cardiolipin (CL) [29, 30] and phosphatidylethanolamine (PE) were used to detect the presence of IgG and IgM antibodies [30]. Values > 10 G phospholipids for anti-CL IgG, > 8 M phospholipids for anti-CL IgM, > 0.3 (95^{th} percentile) for anti-PE IgG, and > 0.45 (95^{th} percentile) for anti-PE IgM were considered positive [31]. These tests were performed twice with a 12-week interval, and if both replicates of a test exceeded the threshold value, it was considered positive. ELISA were performed by a commercial laboratory (SRL, Tokyo, Japan).

Heparin and aspirin treatments

All patients who participated were administered low dose aspirin (81 mg/d) 2 weeks before their expected menstrual period, and were instructed to continue treatment in the event of a positive pregnancy test. Heparin treatment began immediately after a positive urinary pregnancy test. The patients self-administered 5,000 U of unfractionated heparin (Caprocin®, Sawai Pharmacy, Tokyo, Japan) subcutaneously every 12 h.

Blood sample collection

Blood samples were collected immediately after a positive urinary pregnancy test, immediately prior to heparin treatment. Additional blood samples were drawn 1 week later. Control group samples were collected at the same time points.

ELISA to detect serum granulysin

Serum granulysin concentrations were assayed by a commercial laboratory (BML, Tokyo, Japan). Briefly, microtiter plates (Nunc, Roskilde, Denmark) were coated with 5 mg/mL anti-granulysin monoclonal (m)Ab RB1(Mouse IgG1κ) (MBL International Corporation, Nagoya, Japan) in 100 mM carbonate buffer and maintained overnight at 4 °C. The plates were washed with phosphate-buffered saline containing 0.1% Tween 20 (washing buffer) and blocked with 10% fetal bovine serum wash buffer (blocking buffer) for 1–2 h at 37 °C. The plates were then serially reacted at room temperature with the following materials, with one wash in washing buffer between two reactions: samples or standards in blocking buffer for 2 h, 0.1 mg/mL of mouse monoclonal anti-human granulysin biotinylated RC8 mAb (MBL International Corporation) in blocking buffer for 1 h, and 0.05 U/mL of β-galactosidase-conjugated streptavidin (Roche Diagnostics, Tokyo, Japan) in washing buffer for 1 h. After a final wash, the plates were incubated with 0.4 mM 4-methylumbelliferyl bD-galactoside (Sigma-Aldrich) in 10 mM sodium phosphate buffer (pH 7.0), supplemented with 0.02% bovine serum albumin, 100 mM NaCl, and 1 mM $MgCl_2$ at 37 °C, for 17 h. The reaction was then terminated by adding 100 mM glycine-NaOH (pH 10.3), and the fluorescence intensity was measured using a Cytofluor Series 4000 Multi-Well Plate Reader (Applied Biosystems, Foster City, CA, USA) at excitation and emission wavelengths of 360 and 460 nm, respectively.

Statistical analysis

Paired data were analyzed using Welch's *t*-tests and paired *t*-tests (two-sided). Fisher's exact probability test was used to test pregnancy outcomes. All statistical analyses were performed using JMP 6.0 software (SAS Institute, USA). Differences with $P < 0.05$ were considered significant.

Results

Patient characteristics

The characteristics of the patients included in this study are outlined in Table 1. There were 81 patients positive for at least one aPL, which corresponded to 57.0% of the total number of women recruited. The positivity rates were 9.9% for the anti-CL IgG antibody, 7.8% for the anti-CL IgM antibody, 24.1% for the anti-PE IgG antibody, and 41.1% for the anti-PE IgM antibody. The mean gestational age at initial blood sampling was 5.8 ± 1.3 weeks in the aspirin plus heparin group and 6.0 ± 1.9 weeks in the aspirin group; these results were not significantly different.

Table 1
Patient characteristics

Characteristic	Value
Age[a]	35.1 ± 4.1 (23–44)
No. of miscarriages[a]	2.68 ± 1.22 (2–7)
No. of patients with one or more APAs (%)	81 (57.0)
Anti CL IgG positive	14 (9.9)
Anti CL IgM positive	11 (7.8)
Anti PE IgG positive	34 (24.1)
Anti PE IgM positive	58 (41.1)
Abbreviations: *APAs* Antiphospholipid Antibodies, *Anti CL IgG* Anti-cardiolipin IgG, *Anti CL IgM*, Anti-cardiolipin IgM *Anti PE IgG* Anti-phosphatidylethanolamine IgG, *Anti PE IgM*, Anti-phosphatidylethanolamine IgM	
[a]Values are presented in terms of mean ± S.D. (range)	

Serum Granulysin Levels In Women With And Without Apls

Serum granulysin concentrations were significantly higher in women who tested positive for one or more aPLs than in women who tested negative for them (2.75 ± 1.03 ng/mL vs. 2.44 ± 0.69 ng/mL; $P = 0.0341$). There were no significant differences in the granulysin concentrations of women with and without anti-CL IgG or IgM antibodies; however, the concentrations were significantly higher in women who tested positive for either anti-PE IgG or IgM antibodies (Table 2).

Table 2
Serum granulysin concentration in women with or without antiphospholipid antibodies

	Positive (n)	Negative (n)	P value
Antiphospholipid antibodies	2.75 ± 1.03 (81)	2.44 ± 0.69 (60)	0.0341[a]
Anti CL IgG	2.56 ± 1.40 (14)	2.62 ± 0.85 (127)	0.879[a]
Anti CL IgM	2.39 ± 1.04 (11)	2.63 ± 0.90 (130)	0.390[b]
Anti PE IgG	2.98 ± 1.09 (34)	2.51 ± 0.86 (107)	0.013[b]
Anti PE IgM	2.85 ± 1.09 (58)	2.47 ± 0.77 (83)	0.024[a]
Abbreviations: *Anti CL IgG* Anti-cardiolipin IgG, *Anti CL IgM* Anti-cardiolipin IgM *Anti PE IgG* Anti-phosphatidylethanolamine IgG, *Anti PE IgM* Anti-phosphatidylethanolamine IgM			
[a]Welch's *t*-test, [b]Student's *t*-test			

Characteristics of patients treated with aspirin and heparin plus aspirin

Thirty-two women received aspirin plus heparin and thirty received aspirin alone. The mean ages of the aspirin plus heparin and aspirin alone groups were 35.5 ± 3.84 (range: 30–41) years and 33.2 ± 2.94 (range: 29–38) years, respectively, which were not significantly different (Table 3).

Table 3
Characteristics of patients treated with heparin and aspirin and aspirin alone

	Heparin + Aspirin	Aspirin	
No. of patients	32	30	ns
Age[a]	35.5 ± 3.84 (30–41)	33.2 ± 2.94 (29–38)	ns
No. of miscarriages[a]	2.87 ± 1.41 (2–6)	2.47 ± 0.92 (2–5)	ns
[a]Values are presented in terms of mean ± S.D. (range)			
ns: Not significant			

Changes In Serum Granulysin Concentration After Heparin Treatment

The mean serum granulysin level before heparin treatment was 2.77 ± 0.82 ng/mL, which decreased to 2.37± 0.67 ng/mL 1 week after the initiation of heparin treatment. The reduction was statistically significant (P = 0.0007, Fig. 1A). The serum granulysin levels in the aspirin group did not alter significantly

during this period ($P = 0.84$, Fig. 1B). However, no changes in NK cell activity or the type 1/2 helper T cell (Th1/Th2) ratio were observed after heparin treatment (NK cell activity: $P = 0.20$, Th1/Th2: $P = 0.82$; Supplementary Figs. 1 and 2, respectively).

Relationship between pregnancy outcomes and changes in serum granulysin levels

Of the 32 patients treated with heparin plus aspirin, 27 had decreased serum granulysin levels, while five had elevated levels. Miscarriages occurred in 80% (4/5) of those with increased granulysin levels and 15% (4/27) of those with decreased granulysin levels. Therefore, when heparin treatment resulted in decreased serum granulysin levels, a significantly lower miscarriage rate was observed compared to that observed when heparin treatment resulted in increased serum granulysin levels ($P = 0.0086$, Fig. 2).

Discussion

In this study, we observed that serum granulysin levels were higher in women positive for aPLs, especially anti-PE antibodies, and that heparin treatment significantly decreased serum granulysin levels. The miscarriage rate was significantly lower in women whose serum granulysin levels were reduced by heparin treatment, indicating that heparin reduces granulysin levels to prevent miscarriage. In this study, the mechanism by which heparin regulated granulysin levels remained unknown.

Although heparin primarily exerts antithrombotic effects [32, 33], it is also reported to exert an apoptotic effect on the placenta and villi [34, 35] and a suppressive effect on complement activation [9]. In recent years, heparin has also been shown to alter the configuration of high mobility group box 1 (HMGB1) by inhibiting its binding to the receptor of advanced glycation end products [36, 37], suppressing binding to toll-like receptors (TLR4), and suppressing inflammatory signals from TLR4 [38–40] on APCs. HMGB1 is an alarmin that is secreted by necrotic cells or passively secreted by immune cells into the extracellular matrix. It activates APCs, including DCs, as well as granulysin [41]. It acts as a damage-associated molecular pattern and induces pre-inflammatory responses [17]. HMGB1 expression is increased in patients with APS [42] as well as in individuals with RPL of unknown etiology [43]. These findings and our results indicate that heparin may block the binding of HMGB1 to its receptor and suppress the production of granulysin from effector cells. Furthermore, recent studies have revealed that granulysin also acts as an alarmin leading to the activation of TLR4 on APCs [20]. Therefore, we suspected that heparin may suppress the immunostimulatory effects of HMGB1 and granulysin.

It is unclear why granulysin levels were not correlated with NK cell activity in the present study. In general, granulysin is produced by CTLs as well as NK and NKT cells. Indeed, we observed that the production of cytotoxic factors (perforin and granzyme) by uterine NKT cells was significantly higher than that by uterine NK cells observed in our previous study [44]. Therefore, NK cell activation may not have decreased in response to heparin treatment because granulysin was produced by NK cells as well as CTLs and NKT cells. It is also unclear why granulysin levels were not correlated with the Th1/Th2 ratio in the present study. The Th1/Th2 ratio is used to evaluate interferon gamma (IFN-γ) levels (corresponding to Th1) and interleukin (IL)-4 levels (corresponding to Th2). However, it is not only IFN-γ that is involved in Th1 but

also other cytokines such as IL-12, tumor necrosis factor-α, and IL-2 [45]. Since Th2 cytokines are also associated with IL-10 and IL-13 expression in addition to IL4 expression [46], their levels cannot be simply evaluated by the Th1/Th2 ratio. In addition, since Th17 and regulatory T cells also share a complex association [47], it is necessary to investigate the correlation between various cytokines and granulysin in future studies. In this study, we analyzed the NK cell activity and Th1/Th2 ratio in the peripheral blood of patients. In our previous study, we observed the elevation in the levels of cytotoxic granules and inflammatory cytokines, such as IL-2 and IL-12, as well as in the levels of perforin and granzyme B, at the implantation site rather than systemically in murine miscarriage induced by α-galactosylceramide (α-GalCer) [48]. Nakashima et al. also reported that granulysin produced by uterine NK cells induces the apoptosis of extravillous trophoblasts [25], and 85% of NK cells in the decidua of the uterus contain granulysin, which is twice the proportion observed in the periphery [49]. In future, it will be necessary to investigate local changes in these cells in aPL-positive patients.

We previously reported that activated DCs take up α-GalCer and activate NKT cells via CD1d and IL-12, which results in a miscarriage [48]. In this study, the abundance of uterine NKT cells was observed to increase after miscarriage. These cells express multiple cytokines, such as IL-2 and IFN-γ, along with perforin and granzyme, at high levels. However, the actual effector has not been identified. NKT cells produce granulysin [11], which has been reported to induce miscarriage [26]. Therefore, α-GalCer-induced sterile inflammatory miscarriage may be associated with granulysin expression. The alarmin activity of granulysin [15] may also play a role in this. Alarmins stimulate APCs, and granulysin, which is present at high levels in patients with APS (especially those who test positive for anti-PE antibodies), activates PRR-expressing DCs, which may further enhance granulysin secretion by NKT cells, NK cells, and CTLs, and eventually induce miscarriage via its direct effect on apoptosis.

In an earlier study, patients with APS who tested positive for anti-PE antibody showed high granulysin levels, and when this persisted for more than 12 weeks, the miscarriage rate was found to be high [50]. PE is an anti-inflammatory lipid [51]; therefore, anti-PE antibody-positive patients may show higher levels of inflammation, increased immune activity (including granulysin production), and a higher risk of miscarriage. Further research will be required to investigate this.

Serum granulysin concentration may be useful as a clinical marker for RPL. In our study, if heparin treatment reduced serum granulysin levels, the miscarriage rate was found to be lower than if the levels increased. PE antibody-positive patients had high granulysin levels; therefore, it is possible that granulysin is high in “seronegative” APS. In future studies, we intend to evaluate the effects of granulysin on seronegative APS, for which no treatment has been established, and test the effectiveness of heparin.

Preimplantation genetic testing for aneuploidy is being increasingly performed on patients with RPL, which has helped reduce the proportion of miscarriages of unknown etiologies. However, cases of recurrent miscarriage of unknown etiology continue to be reported, including some cases caused by immunological abnormalities. We have already reported the involvement of anti-C9 antibodies in patients of unknown etiology of recurrent miscarriage [52]. In future, we intend to increase the number of

granulysin measurements recorded, establish key clinical thresholds, appropriately identify patients who require heparin, and treat them to reduce the risk of miscarriage. A limitation of this study is its relatively small sample size, which precluded the investigation of anti-β2GPI antibodies or other APS-related antibodies. We intend to increase the sample size in future studies and examine the associations between serum granulysin levels and multiple antibodies.

Conclusion

Serum granulysin levels upregulated in aPL-positive patients were downregulated in response to heparin treatment. This suggests that aPLs are associated with the immune system, and heparin plays a role in inhibiting the immune response.

Abbreviations

α-GalCer
α-Galactosylceramide, APCs:Antigen-Presenting Cells, aPLs:antiphospholipid antibodies, APS:Antiphospholipid antibody Syndrome, β2GPI:β2 glycoprotein I, CL:Cardiolipin, CTLs:Cytotoxic T Lymphocytes, DCs:Dendritic Cells, ELISA:Enzyme-Linked Immunosorbent Assays, HMGB1:High-Mobility Group Box 1, IFN-γ:Interferon Gamma, IL:Interleukin, NK:Natural Killer, NKT:Natural Killer T, NETs:Neutrophil Extracellular Traps, PRRs:Pattern Recognition Receptors, PE:Phosphatidylethanolamine, RPL:Recurrent Pregnancy Loss, TLR4:Toll-like Receptors.

Declarations

Ethics approval and consent to participate

The Ethics Committee of Nippon Medical School Hospital approved the collection and use of biological materials for this study, and all experiments were performed according to the guidelines (19-03-56). This study involved the analysis of human blood samples. Written informed consent was obtained from all study participants.

Consent for publication

All authors consent to the publication of this paper.

Competing interests

The authors declare that they have no competing interest.

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Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection were performed by Tomoko Ichikawa, Mirei Yonezawa, Sayuri Kasano, Ryoko Yokote, and Nozomi Ouchi. Tomoko Ichikawa, Toshiyuki Takeshita, Yasuyuki Negishi, and Yoshimitsu Kuwabara were involved in the data analysis. The first draft of the manuscript was written by Toshiyuki Takeshita and Tomoko Ichikawa. All authors critically revised the manuscript, commented on drafts of the manuscript. All authors read and approved the final manuscript.

Availability of data and material

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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References

1. Di Simone N, Luigi MP, Marco D, Fiorella DN, Silvia D, Clara DM, Alessandro C. Pregnancies complicated with antiphospholipid syndrome: the pathogenic mechanism of antiphospholipid antibodies: a review of the literature. Ann N Y Acad Sci. 2007;1108:505–14.
2. Greaves M. Antiphospholipid syndrome: state of the art with emphasis on laboratory evaluation. Haemostasis. 2000;30 Suppl 2:16–25.
3. De Wolf F, Carreras LO, Moerman P, Vermylen J, Van Assche A, Renaer M. Decidual vasculopathy and extensive placental infarction in a patient with repeated thromboembolic accidents, recurrent fetal loss, and a lupus anticoagulant. Am J Obstet Gynecol. 1982;142(7):829–34.
4. Girardi G, Berman J, Redecha P, Spruce L, Thurman JM, Kraus D, Hollmann TJ, Casali P, Caroll MS, Wetsel RA, Lambris JD, Holters VM, Salmon JE. Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. J Clin Invest. 2003;112(11):1644–54.
5. Yalavarthi S, Gould TJ, Rao AN, Mazza LF, Morris AE, Núñez-Álvarez C, Hernández-Ramírez D, Bockenstedt PL, Liaw PC, Cabral AR, Knight JS. Release of neutrophil extracellular traps by neutrophils stimulated with antiphospholipid antibodies: a newly identified mechanism of thrombosis in the antiphospholipid syndrome. Arthritis Rheumatol. 2015;67(11):2990–3003.
6. Meng H, Yalavarthi S, Kanthi Y, Mazza LF, Elfline MA, Luke CE, Pinsky DJ, Henke PK, Knight JS. In Vivo Role of Neutrophil Extracellular Traps in Antiphospholipid Antibody-Mediated Venous Thrombosis. Arthritis Rheumatol. 2017;69(3):655–67.
7. Laird SM, Tuckerman EM, Cork BA, Linjawi S, Blakemore AI, Li TC. A review of immune cells and molecules in women with recurrent miscarriage. Hum Reprod Update. 2003;9(2):163–74.

8. Guerin J, Sheng Y, Reddel S, Iverson GM, Chapman MG, Krilis SA. Heparin inhibits the binding of beta 2-glycoprotein I to phospholipids and promotes the plasmin-mediated inactivation of this blood protein. Elucidation of the consequences of the two biological events in patients with the anti-phospholipid syndrome. J Biol Chem. 2002;277(4):2644–9. M110176200 [pii].
9. Girardi G, Redecha P, Salmon JE. Heparin prevents antiphospholipid antibody-induced fetal loss by inhibiting complement activation. Nat Med. 2004;10(11):1222–6.
10. Krensky AM, Clayberger C. Granulysin: a novel host defense molecule. Am J Transplant. 2005;5(8):1789–92.
11. Gansert JL, Kiessler V, Engele M, Wittke F, Rollinghoff M, Krensky AM, Porcelli SA, Modlin RL, Stenger S. Human NKT cells express granulysin and exhibit antimycobacterial activity. J Immunol. 2003;170(6):3154–61.
12. Ogawa K, Takamori Y, Suzuki K, Nagasawa M, Takano S, Kasahara Y, Nakamura Y, Kondo S, Sugamura K, Nakamura M, Nagata K. Granulysin in human serum as a marker of cell-mediated immunity. Eur J Immunol. 2003;33(7):1925–33.
13. Clayberger C, Krensky AM. Granulysin. Curr Opin Immunol. 2003;15(5):560–5.
14. Pena SV, Krensky AM. Granulysin, a new human cytolytic granule-associated protein with possible involvement in cell-mediated cytotoxicity. Semin Immunol. 1997;9(2):117–25.
15. Sparrow E, Bodman-Smith MD. Granulysin: The attractive side of a natural born killer. Immunol Lett. 2020;217:126–32.
16. Roh JS, Sohn DH. Damage-Associated Molecular Patterns in Inflammatory Diseases. Immune Netw. 2018;18(4):e27.
17. Cai J, Wen J, Bauer E, Zhong H, Yuan H, Chen AF. The Role of HMGB1 in Cardiovascular Biology: Danger Signals. Antioxid Redox Signal. 2015;23(17):1351–69.
18. Clayberger C, Finn MW, Wang T, Saini R, Wilson C, Barr VA, Sabatino M, Castiello L, Stroncek D, Krensky AM. 15 kDa granulysin causes differentiation of monocytes to dendritic cells but lacks cytotoxic activity. J Immunol. 2012;188(12):6119–26.
19. Castiello L, Stroncek DF, Finn MW, Wang E, Marincola FM, Clayberger C, Krensly AM, Sabatino M. 15 kDa Granulysin versus GM-CSF for monocytes differentiation: analogies and differences at the transcriptome level. J Transl Med. 2011;9:41.
20. Tewary P, Yang D, de la Rosa G, Li Y, Finn MW, Krensky AM, Clayberger C, Oppnheim JJ. Granulysin activates antigen-presenting cells through TLR4 and acts as an immune alarmin. Blood. 2010;116(18):3465–74.
21. Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, Ganz T, Thoma-Uszynski S, Melián A, Bogdan C, Porcelli SA, Bloom BR, Krensky AM, Modlin RL. An antimicrobial activity of cytolytic T cells mediated by granulysin. Science. 1998;282(5386):121–5.
22. Nagasawa M, Ogawa K, Imashuku S, Mizutani S. Serum granulysin is elevated in patients with hemophagocytic lymphohistiocytosis. Int J Hematol. 2007;86(5):470–3.

23. Mincheva-Nilsson L, Nagaeva O, Sundqvist KG, Hammarstrom ML, Hammarstrom S, Baranov V. gammadelta T cells of human early pregnancy decidua: evidence for cytotoxic potency. Int Immunol. 2000;12(5):585–96.
24. Sakai M, Ogawa K, Shiozaki A, Yoneda S, Sasaki Y, Nagata K, Saito S. Serum granulysin is a marker for Th1 type immunity in pre-eclampsia. Clin Exp Immunol. 2004;136(1):114–9.
25. Nakashima A, Shiozaki A, Myojo S, Ito M, Tatematsu M, Sakai M, Takamori Y, Ogawa K, Nagata K, Saito S. Granulysin produced by uterine natural killer cells induces apoptosis of extravillous trophoblasts in spontaneous abortion. Am J Pathol. 2008;173(3):653–64.
26. Gulic T, Laskarin G, Dominovic M, Glavan Gacanin L, Babarović E, Rubesa Z, Haller H, Rukavina D. Granulysin-mediated apoptosis of trophoblasts in blighted ovum and missed abortion. Am J Reprod Immunol. 2018;80(3):e12978.
27. Fujioka T. Introducing the Hemos Iel Thrombophilia Screening Panel. Igaku to Yakugaku. 2016:621–7.
28. Pengo V, Tripodi A, Reber G, Rand JH, Ortel TL, Galli M, De Groot PG. Update of the guidelines for lupus anticoagulant detection. Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis. J Thromb Haemost. 2009;7(10):1737–40.
29. Okada J. Usefulness of the MESACUP cardiolipin test. Igaku and Yakugaku. 1996:1389–94.
30. Junichi Kaburagi KO. Measuring IgM anti-cardiolipin antibodyDevelopment of enzyme-linked immunosorbent assay (ELISA) and its clinical usefulness. Igaku and Yakugaku, 2000:1183–8.
31. Sugi T, Katsunuma J, Izumi S, McIntyre JA, Makino T. Prevalence and heterogeneity of antiphosphatidylethanolamine antibodies in patients with recurrent early pregnancy losses. Fertil Steril. 1999;71(6):1060–5.
32. Bates SM, Rajasekhar A, Middeldorp S, McLintock C, Rodger MA, James AH, Vazquez SR, Greer IA, Riva JJ, Bhatt M, Schwab N, Barrett D, LaHaye A, Rochwerg B. American Society of Hematology 2018 guidelines for management of venous thromboembolism: venous thromboembolism in the context of pregnancy. Blood Adv. 2018;2(22):3317–59.
33. Bates SM, Greer IA, Middeldorp S, Veenstra DL, Prabulos AM, Vandvik PO. VTE, thrombophilia, antithrombotic therapy, and pregnancy: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. Chest. 2012;141(2 Suppl):e691S–e736S.
34. Bose P, Black S, Kadyrov M, Weissenborn U, Neulen J, Regan L, Huppertz, B. Heparin and aspirin attenuate placental apoptosis in vitro: implications for early pregnancy failure. Am J Obstet Gynecol. 2005;192(1):23–30.
35. Hills FA, Abrahams VM, González-Timón B, Francis J, Cloke B, Hinkson L Rai R, Mor G, Regan L, Sullivan M, Lam EWF, Brosens JJ. Heparin prevents programmed cell death in human trophoblast. Mol Hum Reprod. 2006;12(4):237–43.

36. Ling Y, Yang ZY, Yin T, Li L, Yuan WW, Wu HS, Wang CY. Heparin changes the conformation of high-mobility group protein 1 and decreases its affinity toward receptor for advanced glycation endproducts in vitro. Int Immunopharmacol. 2011;11(2):187–93.
37. Zenerino C, Nuzzo AM, Giuffrida D, Biolcati M, Zicari A, Todros T, Rolfo A. The HMGB1/RAGE Pro-Inflammatory Axis in the Human Placenta: Modulating Effect of Low Molecular Weight Heparin. Molecules. 2017;22(11).
38. Babazada H, Yanamoto S, Hashida M, Yamashita F. Binding and structure-kinetic relationship analysis of selective TLR4-targeted immunosuppressive self-assembling heparin nanoparticles. Int J Pharm. 2018;552(1–2):76–83.
39. Li X, Liu Y, Ma X. Unfractionated heparin inhibits lipopolysaccharide-induced expression of granulocyte colony-stimulating factor in human endothelial cells through Toll-like receptor 4 signaling pathway. Zhonghua Wei Zhong Bing Ji Jiu Yi Xue. 2015;27(2):81–5.
40. Babazada H, Yamashita F, Hashida M. Suppression of experimental arthritis with self-assembling glycol-split heparin nanoparticles via inhibition of TLR4-NF-κB signaling. J Control Release. 2014;194:295–300.
41. van Beijnum JR, Buurman WA, Griffioen AW. Convergence and amplification of toll-like receptor (TLR) and receptor for advanced glycation end products (RAGE) signaling pathways via high mobility group B1 (HMGB1). Angiogenesis. 2008;11(1):91–9.
42. Manganelli V, Truglia S, Capozzi A, Alessandri C, Riitano G, Spinelli FR, Ceccarelli F, Mancuso S, Garofalo T, Longo A, Valesini G, Sorice M, Conti F, Misasi R. Alarmin HMGB1 and Soluble RAGE as New Tools to Evaluate the Risk Stratification in Patients With the Antiphospholipid Syndrome. Front Immunol. 2019;10:460.
43. Zou H, Yin J, Zhang Z, Xiang H, Wang J, Zhu D, Xu X, Cao Y. Destruction in maternal-fetal interface of URSA patients via the increase of the HMGB1-RAGE/TLR2/TLR4-NF-κB signaling pathway. Life Sciences. 2020;250: 117543.
44. Negishi Y, Ichikawa T, Takeshita T, Takahashi H. Miscarriage induced by adoptive transfer of dendritic cells and invariant natural killer T cells into mice. Eur J Immunol. 2018;48(6):937–49.
45. Romagnani S. Th1/Th2 cells. Inflamm Bowel Dis. 1999;5(4):285–94.
46. Romagnani S. T-cell subsets (Th1 versus Th2). Ann Allergy Asthma Immunol. 2000;85(1):9–18.
47. Morita K, Tsuda S, Kobayashi E, Hamana H, Tsuda K, Shima T, Nakashima A, Ushijima A, Kishi H, Saito S. Analysis of TCR Repertoire and PD-1 Expression in Decidual and Peripheral $CD8^+$ T Cells Reveals Distinct Immune Mechanisms in Miscarriage and Preeclampsia. Front Immunol. 2020;11:1082.
48. Ichikawa T, Negishi Y, Shimizu M, Takeshita T, Takahashi H. α-Galactosylceramide-activated murine NK1.1(+) invariant-NKT cells in the myometrium induce miscarriages in mice. Eur J Immunol. 2016;46(8):1867–77.
49. Vujaklija DV, Gulic T, Sucic S, Nagata K, Ogawa K, Laskarin G, Saito S, Haller H, Rukavina D. First trimester pregnancy decidual natural killer cells contain and spontaneously release high quantities of

granulysin. Am J Reprod Immunol. 2011;66(5):363–72.

50. Yonezawa M, Kuwabara Y, Ono S, Ouchi N, Ichikawa T, Takeshita T. Significance of Anti-Phosphatidylethanolamine Antibodies in the Pathogenesis of Recurrent Pregnancy Loss. Reprod Sci. 2020;27(10):1888–93.
51. Ireland R, Schwarz B, Nardone G, Wehrly TD, Broeckling CD, Chiramel AI, Best, SM, Bosio, CM. Unique Francisella Phosphatidylethanolamine Acts as a Potent Anti-Inflammatory Lipid. J Innate Immun. 2018;10(4):291–305.
52. Kuwabara Y, Katayama A, Kurihara S, Orimo H, Takeshita T. Immunoproteomic identification of anti-C9 autoimmune antibody in patients with seronegative obstetric antiphospholipid syndrome. PLoS One. 2018;13(6):e0198472.

Figures

Figure 1

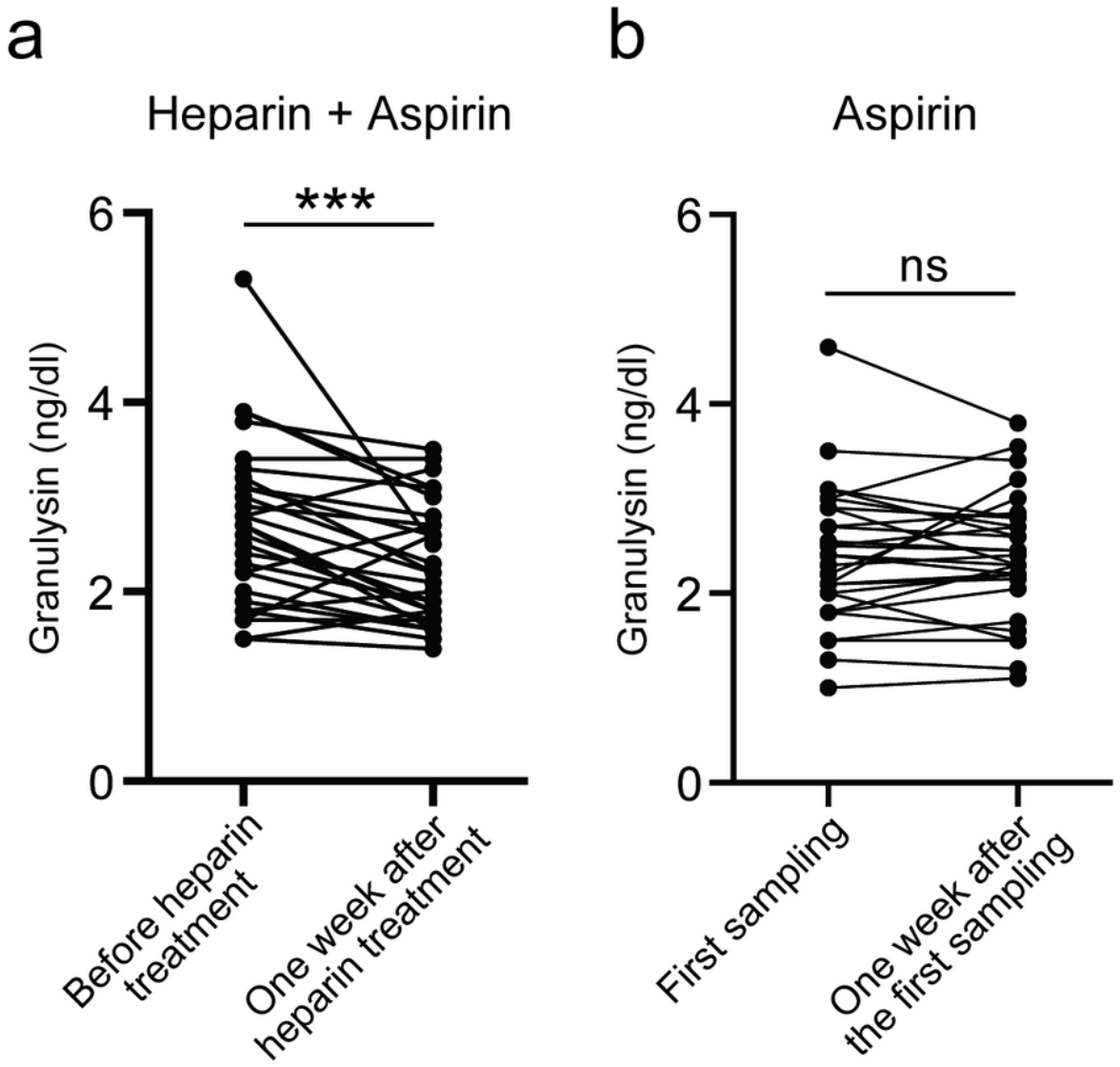

Figure 1

Changes in serum granulysin concentration for the patients with RPL. Serum granulysin concentration was assayed using ELISA. (a) Serum granulysin levels for the patients treated with combination therapy of aspirin and heparin were measured before and 1 week after the initiation of heparin treatment (n = 32). (b) Serum granulysin levels for the patients treated with aspirin alone (n = 30). The gestational age of sampling is the same as in the aspirin heparin combination therapy group. ***P <0.001: Paired t-test

Figure 2

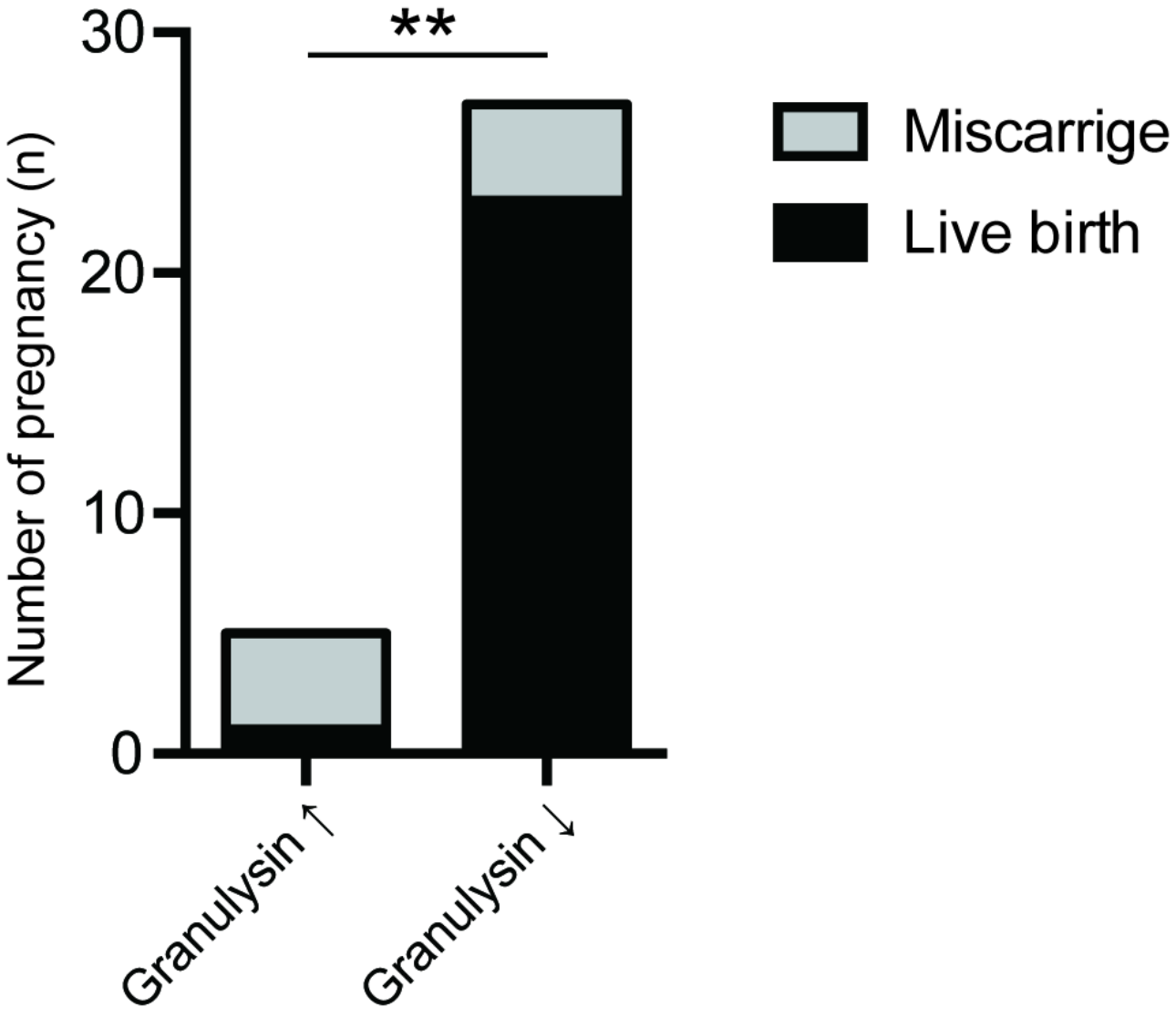

Figure 2

Pregnancy outcome stratified by granulysin levels after heparin treatment. **P <0.05; Fisher's exact probability test

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