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Anti-phospholipid induced murine fetal loss: Novel protective effect of a peptide targeting the β2 glycoprotein I phospholipid-binding site. Implications for human fetal loss

Yeny Martinez de la Torre [a,1], Francesca Pregnolato [b], Fabio D'Amelio [c], Claudia Grossi [b], Nicoletta Di Simone [d], Fabio Pasqualini [e], Manuela Nebuloni [f], Pojen Chen [g], Silvia Pierangeli [h], Niccolò Bassani [i], Federico Ambrogi [i], Maria-Orietta Borghi [b,c], Annunciata Vecchi [e], Massimo Locati [a,j], Pier-Luigi Meroni [b,c,k,*]

[a] Istituto Clinico Humanitas, Rozzano, Italy
[b] Istituto Auxologico Italiano, Milan, Italy
[c] Department of Internal Medicine, University of Milan, Italy
[d] Catholic University of Sacred Heart, Rome, Italy
[e] Fondazione Humanitas per la Ricerca, Rozzano, Italy
[f] L. Sacco Department of Medical Science, University of Milan, Italy
[g] Department of Medicine, University of Los Angeles, CA, USA
[h] Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX, USA
[i] Department of Work Medicine, University of Milan, Italy
[j] Department of Translational Medicine, University of Milan, Italy
[k] Division of Rheumatology, Istituto G. Pini, Piazza Ferrari 1, 20122 Milan, Italy

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ABSTRACT

β2 glycoprotein I (β2GPI)-dependent anti-phospholipid antibodies (aPL) induce thrombosis and affect pregnancy. The CMV-derived synthetic peptide TIFI mimics the PL-binding site of β2GPI and inhibits β2GPI cell-binding *in vitro* and aPL-mediated thrombosis *in vivo*. Here we investigated the effect of TIFI on aPL-induced fetal loss in mice. TIFI inhibitory effect on *in vitro* aPL binding to human trophoblasts was evaluated by indirect immunofluorescence and ELISA. TIFI effect on aPL-induced fetal loss was investigated in pregnant C57BL/6 mice treated with aPL or normal IgG (NHS). Placenta/fetus weight and histology and RNA expression were analyzed. TIFI, but not the control peptide VITT, displayed a dose-dependent inhibition of aPL binding to trophoblasts *in vitro*. Injection of low doses of aPL at day 0 of pregnancy caused growth retardation and increased fetal loss rate, both significantly reduced by TIFI but not VITT. Consistent with observations in humans, histological analysis showed no evidence of inflammation in this model, as confirmed by the absence of an inflammatory signature in gene expression analysis, which in turn revealed a TIFI-dependent modulation of molecules involved in differentiation and development processes. These findings support the non-inflammatory pathogenic role of aPL and suggest innovative therapeutic approaches to aPL-dependent fetal loss.

1. Introduction

Anti-phospholipid antibodies (aPL) represent the most frequent acquired risk factor for a treatable cause of recurrent pregnancy loss and complications [1]. Such a clinical association is also supported by experimental models showing that passive transfer of aPL induces fetal loss and growth retardation in pregnant naive mice [2]. The pathogenic mechanisms are still matter of debate [2,3] as well as the treatment since the standard therapy (low dose aspirin and heparin) does not protect all the patients from recurrent miscarriages [4].

Although intraplacental thrombosis with impairment of maternal–fetal blood exchange was thought to play a role, histopathological findings suggestive for thrombosis cannot be detected in the majority of miscarriage samples and placentas from anti-

* Corresponding author. Division of Rheumatology, Istituto G. Pini, Piazza Ferrari 1, 20122 Milan, Italy. Tel.: +39 02 58296746; fax: +39 02 58318176.
E-mail address: pierluigi.meroni@unimi.it (P.-L. Meroni).
[1] Present address: Department of Clinical Chemistry, School of Medicine, University of Crete, Heraklion, Greece.

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phospholipid syndrome (APS) women [2,5,6]. Accordingly, alternative mechanisms have been suggested. For example, passive infusion of large amounts of IgG aPL in naive mice after embryo implantation may induce fetal loss via placental inflammation [7–12]. Again, these experimental observations do not find correspondence in immunohistological analysis of abortive material or term placentas from APS women which does not offer conclusive information on the pathogenic contribution of acute local inflammation and complement deposition [6,13–15]. Evidence has been collected for alternative aPL-mediated non-thrombotic/non-inflammatory mechanisms ending into a defective placentation [2,3,5,12,16]. Consistent with this, an inflammatory process does not appear to be involved in an alternative model of fetal resorption based on i.v. injection of smaller amount of human aPL into mice before implantation. This model is much closer to the human disease where the autoantibodies are present even before conception and at low levels [17,18]. As a whole these findings suggest that the pathogenesis of APS-associated pregnancy complications may be more heterogeneous than expected [2,3,5,19].

Irrespective to the underlying molecular mechanisms, the expression of β2GPI at placental level is the cornerstone to explain the β2GPI-aPL pathogenic role. β2GPI may bind to placental tissues via several cell receptors and a localized expression of the molecule was reported both in normal human placentas and at the embryo implantation sites in experimental models [2,20]. The synthetic peptide TIFI, spanning Thr(101)–Thr(120) of ULB0-HCMVA from human CMV, shares similarity with the β2GPI PL-binding site. This peptide prevents aPL-mediated thrombosis *in vivo* and inhibits the *in vitro* binding of FITC-conjugated β2GPI to human endothelial cells and murine monocytes [21]. Since aPL do not react with TIFI, the inhibitory effect was thought to result from its ability to compete with the β2GPI PL-binding site and to displace the molecule from the cell surfaces, ultimately inhibiting aPL binding to the target tissues [21].

With this as background, we hypothesized that TIFI may be a novel therapeutic tool able to inhibit β2GPI binding to trophoblast and to be protective against aPL-mediated placental damage. In addition, the inhibitory effect may provide further insights on the nature of the involved non-thrombotic/non-inflammatory pathogenic mechanisms.

2. Materials and methods

2.1. Reagents

Polyclonal IgG were purified from the sera of 5 APS patients (aPL) diagnosed according to the Sidney criteria [1] and displaying medium-high titer of anti-cardiolipin and anti-β2GPI antibodies as described [22] (Table 1 Supplemental File). Control IgG were from 5 aPL-negative blood donors (NHS). The final IgG concentration, their reactivity with cardiolipin or β2GPI-coated plates, and the endotoxin contamination were evaluated as previously described [22]. The human IgG anti-β2GPI monoclonal antibody (moAb) IS3 was obtained from an APS patient as described [23] and purified from the culture supernatants. A human moAb of irrelevant specificity was used as control. Human β2GPI was purified from human serum and characterized as previously described [20,22]. Sequence for the TIFI peptide, spanning Thr101–Thr120 of the human CMV ULB0 protein, and the control peptide VITT, spanning Val51–Ile70 of the human CMV US27 protein, were obtained from Swiss Protein Database Designation. Both share structural similarity with the 15-aminoacid peptide called GDKV in the fifth domain of human β2GPI, but display opposite effects *in vivo* [21,24].

2.2. Trophoblast cell cultures and binding assay

Placentas were obtained from healthy women immediately after uncomplicated vaginal delivery at ≥36 week gestation. Cytotrophoblast cells were isolated, cultured and characterized as described [25]. Ninety-five percent of the cell preparations tested positive for anti-cytokeratin antibodies. Cytotrophoblasts at different times of culture were further assayed for the cytoplasmic presence of human chorionic gonadotrophin as a marker for syncytiotrophoblast. For binding assay, the trophoblast monolayer was washed three times with HBSS (Sigma Aldrich) and cultured in FBS-free medium to remove adherent β2GPI. FBS-free medium trophoblast cells were then incubated for 1 h with exogenous human β2GPI (5 μg/ml). Polyclonal or monoclonal anti-β2GPI antibodies (50 or 25 μg/ml, respectively) were added to the wells in the presence or absence of serial concentrations of TIFI or VITT. After 2 h of incubation the antibody binding was detected as described [25]. The binding was also evaluated by indirect immunofluorescence in comparable experimental conditions using a FITC-labeled goat anti-human IgG as a secondary antibody (Sigma Aldrich).

2.3. Animals and experimental models

C57BL/6 mice (7–8 weeks old) from Charles River Italia were used in accordance with institutional guidelines in compliance with national and international law and policies [17]. The day of vaginal plug detection, day 0 of pregnancy, mice were infused i.v. with aPL (10–50–100 μg/mouse/200 μl PBS) or NHS. On days 0, 5, and 10 mice were treated i.p. with 40 μg/mouse of TIFI or VITT in PBS or with PBS alone (200 μl/mouse). On day 0 peptides were given 30 min before aPL or NHS injection (50 μg/mouse). Mice were sacrificed on day 15, embryonic sacs removed and weighted, then opened and fetuses and placentas dissected and individually weighted. Reabsorbed fetuses were identified by their small size and necrotic or hemorrhagic appearance compared with normal embryos. Results are presented as the percentage of fetal loss – calculated as reported – and as weights [17,18].

2.4. Histology

Murine placentas were fixed in 10% buffered formalin for 24–48 h and paraffin embedded. Longitudinal sections of 3 μm were stained with hematoxylin–eosin and histological examination was performed in a blinded fashion by a pathologist.

2.5. Gene expression analysis

Total RNA was purified from homogenized murine placental tissues by TRIzol Reagent (Invitrogen), treated with DNase (Applied Biosystem), and assayed for quality by a BioPhotometer Plus (Eppendorf) and electrophoresis. Gene expression profile was analyzed by MouseWG-6 v2 Expression BeadChip kit (Illumina) for the evaluation of the whole mouse genome expression, according to manufacturer's protocol. Four different RNA samples were tested for mice treated with aPL and aPL + TIFI, while three samples were assayed for the NHS group. Array normalization was performed within the BeadStudio v.3 software using the Quantile method. Referring to the Chip version v2, probes mapping to the same gene according to the official Gene Symbol were averaged. Intensity data were filtered on a detection p-value > 0.1 on all samples, assuming that they reflected experimental noise and carried no relevant biological information, and log2 transformed. Differential expression analysis was performed within JMP Genomics 4.1 software, by fitting a gene-wise linear model using treatment group as only covariate and exploring relevant contrasts, evaluated using an empirical Bayes