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Gene expression profiling identifies distinct molecular signatures in thrombotic and obstetric antiphospholipid syndrome

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ABSTRACT

Antiphospholipid antibodies (aPL) cause vascular thrombosis (VT) and/or pregnancy morbidity (PM). Differential mechanisms however, underlying the pathogenesis of these different manifestations of antiphospholipid syndrome (APS) are not fully understood. Therefore, we compared the effects of aPL from patients with thrombotic or obstetric APS on monocytes to identify different molecular pathways involved in the pathogenesis of APS subtypes. VT or PM IgG induced similar numbers of differentially expressed (DE) genes in monocytes. However, gene ontology (GO) analysis of DE genes revealed disease-specific genome signatures. Compared to PM, VT-IgG showed specific up regulation of genes associated with cell response to stress, regulation of MAPK signalling pathway and cell communication. In contrast, PM-IgG regulated genes involved in cell adhesion, extracellular matrix and embryonic and skeletal development. A novel gene expression analysis based on differential variability (DV) was also applied. This analysis identified similar GO categories compared to DE analysis but also uncovered novel pathways modulated solely by PM or VT-IgG. Gene expression analysis distinguished a differential effect of VT or PM-IgG upon monocytes supporting the hypothesis that they trigger distinctive physiological mechanisms. This finding contributes to our understanding of the pathology of APS and may lead to the development of different targeted therapies for VT or PM APS.

1. Introduction

The antiphospholipid syndrome (APS) is an autoimmune disease in which antiphospholipid antibodies (aPL) cause vascular thrombosis and/or pregnancy morbidity. Current APS classification criteria identify the presence of aPL using: anti-cardiolipin antibodies (aCL); anti-beta 2-glycoprotein I antibodies (aβ2GPI); and/or Lupus anticoagulant (LA) assays. The classification criteria for APS identify two major types of clinical events: thrombosis and pregnancy morbidity. Thus, there are three groups of patients with APS – those with a history of vascular thrombosis but not pregnancy morbidity (VT+/PM-), those with pregnancy morbidity but no vascular thrombosis (VT-/PM+) and those who have suffered both types of clinical events (VT+/PM+). Long-term follow-up studies over many years showed that most VT+/PM- patients never develop PM and most VT-/PM+ patients never develop VT [1,2] suggesting that, in these patients, the aPL that cause VT and those that cause PM may bind different antigens and cellular receptors to have diverse effects on target cells.

Many studies have aimed to determine whether specific aPL are associated with thrombotic or obstetric manifestations. One particular study reported LA as the primary predictor of adverse pregnancy outcome [3]. However, no one aPL test has emerged clearly as the leading marker of vascular thrombosis or pregnancy morbidity in APS.

A few studies found differences in the effects of aPL from patients with and without thrombosis upon various signalling pathways in target cells [4–7]. Proteomic analyses of monocytes isolated from patients with APS [8] and healthy monocytes treated with APS-IgG [9] have shown that the monocyte proteome is differentially regulated by IgG from obstetric compared to thrombotic APS.

A limited number of microarray studies in APS have reported differences in gene expression in peripheral blood mononuclear cells (PBMCs) from patients with APS compared to healthy controls (HC) [10]; endothelial cells treated with aPL [11] and monocytes isolated from patients with APS, HC and/or SLE [12]. These studies however,

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did not examine for differences in gene expression between APS subtypes.

In the present study, we carried out microarray analysis comparing patterns of mRNA expression in monocytes from a healthy volunteer exposed to VT+/PM-, VT-/PM+ or HC-IgG. We specifically sought to compare the effects of aPL from patients with different manifestations of APS on monocytes.

Our microarray approach involved the traditional methods of analysis including the identification of genes with significant changes in mean expression level, also known as differentially expressed genes (DE). DE genes were defined as transcripts showing an unusually high or low expression level under a particular treatment compared with transcript expression levels of other genes under the same treatment. The DE genes of particular interest in this study are those which differ between the groups i.e. they are differentially regulated in monocytes treated with either VT+/PM- IgG or VT-/PM+ IgG but not both.

We also employed a novel form of analysis that looks at differential variability (DV) of genes. This analysis is not concerned with the absolute level of expression of particular genes, but the degree to which that level varies amongst cells exposed to different IgG samples from patients of the same phenotype. In the first description of the DV method, Ho et al. [13] argued that DV analysis was biologically relevant and valuable because it gives insight into cellular regulation. DV analysis typically identifies a smaller number of genes than DE analysis and may identify different genes than DE, so that the two types of analysis can be used in parallel as we have done in this paper. We have used both DE and DV analysis of gene expression data obtained by microarray to identify molecular signals and functional pathways that differ between thrombotic and obstetric APS.

2. Methods

2.1. Patients

Serum samples (n = 27) were obtained with informed consent and appropriate local ethical approval. Of 18 patients fulfilling APS classification criteria [14], 3 also had SLE, fulfilling the classification criteria [15] and 15 had primary (P)APS. Healthy individuals (n = 9) were aPL and APS negative.

2.2. Immunological characterisation and purification of IgG

IgG was protein G purified, passed through Endotoxin removal columns (Thermo Scientific) and confirmed to be < 0.06 endotoxin units/ml by EndoLISA® (Hyglos). Concentration was determined by spectrophotometry. IgG aCL and anti-β2GPI titres were determined as previously [16]. Serum LA was measured by dilute Russell's viper venom time.

2.3. Isolation and culture of human monocytes

In order to reduce sample variability, peripheral venous blood samples from a single healthy donor were used to isolate mononuclear blood cells using SepMate tubes (StemCell Technology) and Ficoll-Paque Plus (GE Healthcare). Monocytes were purified using the immunomagnetic Easysep human CD14 + ve selection protocol (StemCell Technology). Monocytes were cultured at 37 °C and 5% CO_2 in RPMI 1640 supplemented with 10% heat-inactivated FBS, 20 U/mL penicillin, 20 μg/mL streptomycin and 2 mM L-glutamine.

2.4. In vitro exposure of monocytes to IgG

For microarray hybridisation, 1×10^6 monocytes were treated with 200 μg/ml of individual IgG from 8 VT+/PM−, 6 VT−/PM+ or 8 HC for six hours. For target validation, 2.5×10^5 monocytes were treated with 200 μg/ml of individual IgG from 9 VT+/PM−, 9 VT−/PM+ or 9 HC for six hours. All IgG samples from APS displayed a higher aCL (> 40 GPLU) and anti-β2GPI (> 10 GBU) binding compared to healthy controls that were negative in these assays.

2.5. RNA extraction, labelling and gene expression analysis

Total RNA from monocytes stimulated with IgG was extracted using the RNeasy mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated with DNase I and used to obtained biotin labelled cRNA (Applied Biosystems). The quality and quantity of cRNA were determined using a NanoPhotometer® (Implen). Gene expression profile was done using HumanHT-12 v4 bead chip array (Illumina) according to manufacturer's protocol. Groups of samples based on IgG source were equally distributed on the two chips to avoid batch effect.

Raw data was generated by Genome Studio and analyzed using R software 3.0.2 (limma and lme 4 packages). Background correction, quantile normalization and log 2 transformation were applied to standardized signal among samples. A linear mixed model (LMM) [17] and a finite mixture model (FMM) were applied to identify DE transcripts. A threshold > 0.8 of the Bayesian posterior probability comparable to a 0.05 False Discovery Rate (FDR) threshold was chosen to reduce false positives.

A DV analysis as described in Ho et al. [13] was also performed. After outlier removal, an F test was applied to look for genes with significant DV. Multiple tests were corrected with a 0.05 FDR procedure.

Gene ontology (GO) enrichment was performed using Panther-GO (http://www.pantherdb.org/) and DAVID (https://david.ncifcrf.gov) bioinformatics tools. In the last case, main biological processes were clustered in a functional annotation analysis with a fold enrichment score based on Expression Analysis Systematic Explorer (EASE) score.

2.6. Validation of data by quantitative real-time PCR (qPCR)

Total RNA from monocytes stimulated with IgG was extracted using the RNeasy mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was analyzed by quantitative RT-PCR using a TaqMan® based assay (Applied Biosystems) in a DNA Engine Opticon continuous fluorescence detector (MJ Research). Gene expression was determined relative to GAPDH using the comparative threshold method. The inventoried Taqman probes used were as follows: PTK2B (Hs00169444_m1), TIPM2 (Hs00234278_m1), GP5 (Hs03027242_s1), FN1 (Hs01549976_m1), C4A (Hs00416393_g1), AKT1 (Hs00178289_m1), CAV1 (Hs00971716_m1), EPOR (Hs00959427_m1), NRP1 (Hs00826128_m1), CCL22 (Hs01574247_m1).

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism software 5.0 (GraphPad Software). Data were first tested for normality and equal variance. If data were normally distributed, comparisons were made using one-way analysis of variance. If data was not normally distributed, a Kruskal-Wallis test was used.

3. Results

3.1. Clinical and laboratory characteristics of individuals

A total of 18 patients with APS (9 VT and 9 PM) and 9 HC subjects were included in the study (Table 1). Twenty-one (78%) of the subjects were female. Of the 18 patients with APS, 3 had SLE and 15 primary APS. Nine patients with VT+/PM- had thrombotic manifestations (6 venous, 6 arterial and 3 recurrent). Seven patients with VT-/PM+ had second trimester foetal losses and two a first trimester foetal loss. Serum and purified IgG from patients in the VT+/PM- and VT-/PM+ groups had higher levels of aCL and anti-β_2GPI activity compared to HC, but