Abstract

Objective. Microarray studies have provided insight into the pathogenesis of systemic JIA and have opened new avenues for therapy. Data on the pathogenesis of the enthesitis-related arthritis (ERA) category of JIA are limited, thus we studied the expression profile of ERA patients' peripheral blood and SF mononuclear cells (PBMCs and SFMCs, respectively). PBMCs from healthy subjects were used as controls.

Methods. RNA from PBMCs of ERA patients (n = 17) and healthy controls (n = 8) and seven ERA SFMCs were converted to labelled cRNA and hybridized to Illumina Human WG-6_v3_BeadChip chips. Expression profiles were analysed using GeneSpring software. Selected genes of interest were validated by real-time PCR.

Results. There was no significant difference in PBMC gene expression of ERA and control groups. However, there was a significant difference between expression profiles of SFMCs and PBMCs of patients with ERA, with 131 genes being overexpressed and 216 being underexpressed in SFMCs. Among genes involved with immune function, cluster of differentiation (CD)1b, CD1d, MHC class II alpha and beta chain, and soluble CD163 were overexpressed, whereas genes related to NK cell function, namely, Granzyme H, killer cell lectin-like receptor subfamily F member 1, killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail (KIR3DL3), natural killer group 7 (NKG7) and other genes like CD244, CD248 and Fas apoptotic inhibitory molecule 3 (FAIM3) were underexpressed.

Conclusion. ERA SFMCs had a distinct gene expression profile from PBMCs and had higher expression of genes associated with antigen presentation, scavenger function, chemotaxis and proteases, whereas genes involved in NK cell function, cell adhesion and inhibitors of apoptosis were underexpressed.

Key words: gene expression, inflammation, JIA, synovial fluid.

Introduction

Enthesitis-related arthritis (ERA)—one of the seven categories of JIA [1]—is characterized by the presence of enthesitis, arthritis, male preponderance and strong association with HLA-B*27 [2]. Autoantibodies and autoreactive T cells are absent in ERA. Hence it is believed that the immune dysregulation is owing to the interplay between genetic and environmental factors [3]. One way to gain information about such a complex disease is to do a whole genome expression profile of affected individuals and identify dysregulated genes and pathways.

Analysis of the transcriptome using microarrays has revealed novel disease-specific signatures in RA, PsA and AS. It was found that the C-X-C chemokine receptor type 4/stromal-derived-factor-1 was a potential pro-inflammatory axis in all three diseases [4]. Gene expression profiling of systemic-onset JIA (SoJIA) resulted in successful use of anakinra to achieve clinical remission in these patients [5]. Subsequently the gene expression signature was shown to distinguish between active and inactive SoJIA patients [6, 7].

Limited data are available on the transcriptional profile in ERA patients. Only two pathways, haemoglobin and immunoglobulin genes, and tyrosine metabolism were reported to be significantly dysregulated in ERA peripheral
blood mononuclear cells (PBMCs) [8]. However, there are no data on gene expression profiling of SF mononuclear cells (SFMCs) from patients with ERA. We hypothesize that studying the SFMC transcriptome may yield clues to mechanisms involved in synovial inflammation and joint pathology. Therefore we compared the gene expression profile of PBMCs and SFMCs.

Materials and methods

Patients and controls

Peripheral blood and SF (when available) were obtained from patients who satisfied ILAR criteria [1] and who or whose parents gave informed consent. Blood was collected from similar age and gender non-related healthy control subjects. Written informed consent was obtained from all patients. The study was approved by the institutional ethics committee (Institutional Ethics Committee for Human Research at Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India).

Isolation of plasma and cell-free SF

Blood and SF were centrifuged at 1000 g for 10 min, within 30 min of withdrawal. Plasma/cell-free SF was stored at −80°C.

Isolation of mononuclear cells

PBMCs and SFMCs were isolated by layering on Histopaque 1077-1 (Sigma-Aldrich, St Louis, MO, USA). Cells were frozen in Trizol at a concentration of 5–7 × 10^6/ml and stored at −80°C.

RNA isolation

RNA was isolated by columns (RNeasy kit, Qiagen, CA, USA) using the manufacturer’s protocol with slight modification. After lysing the cells, 200 μl of chloroform was added per millilitre of Trizol used. After centrifugation at 4°C for 10 min at 12,000 g, the interface was separated and subsequent processing was done using columns. RNA was eluted in RNaše-free water and estimated by spectrophotometer (Nanodrop, Wilmington, DE, USA). RNA integrity was checked using an Agilent Bioanalyser (Agilent Technologies, Inc., Santa Clara, CA, USA). Samples that had 260/280 and 260/230 ratios >2.0 were selected for expression profiling.

Microarray data acquisition and analysis

The gene expression profiling by illumina Human WG-6_v3_BeadChip chips (Illumina, San Diego, CA, USA) was carried out at the Centre for Genomic Applications, New Delhi. RNA was converted into cDNA and biotin-labelled cRNA. The cRNA was randomly hybridized to array and scanned with an Illumina iScan Reader. The array intensity data were analysed using the illumina GenomeStudio Gene Expression Module (v1.1.1) (Illumina, San Diego, CA, USA) for visualization and normalization. The quantile normalization method was used for all analyses and average background correction performed using Beadstudio software. Further analysis was performed in GeneSpring GX version 11.5 software (Agilent Technologies Inc.). Statistical analysis was performed using a t-test with a Benjamini–Hochberg false discovery rate of 5%. Expression profiles were further subjected to hierarchical clustering algorithms and gene ontology analysis.

Validation by quantitative PCR and ELISA

Ct values (ΔCt = Ct gene of interest − Ct GAPDH) of the test sample and calibrator samples were tested for significance as described in the Statistical analysis of validation data section. Relative fold change was determined using the ΔΔCt method [9]. Levels of sCD163 were determined by Duo SET ELISA development kits (R&D Systems, Minneapolis, MN, USA) as per the manufacturer’s instructions.

Statistical analysis of validation data

Data were analysed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Mann-Whitney U-test was used for comparing differences between ERA patients and healthy control subjects, whereas analysis of paired blood and SF samples was done by Wilcoxon’s signed rank test.

Results

Patient demographics are given in supplementary Table 1, available as supplementary data at Rheumatology Online. Nineteen ERA patients were included in the study. Of these, because there were fewer samples, 17 samples were used for the microarray study, whereas real-time PCR was done for all 19. Paired SF was obtained from 10 patients; microarray analysis was done for seven samples and real-time PCR for all. Eight non-related healthy male control subjects with a median age of 23 (20–24) years were included.

Microarray results

Principal component analysis revealed marked heterogeneity between samples (supplementary Fig. 1, available as supplementary data at Rheumatology Online). ERA PB could be distinguished from ERA SF and control PB using supervised clustering, but unsupervised clustering of individual samples revealed that there is coclustering of several unrelated samples, probably because of heterogeneity.

At a cut-off of fold change ≥2 and detection P < 0.05, no difference was observed between ERA PBMCs and
healthy PBMCs. ERA PBMCs had 216 genes upregulated and 131 genes downregulated compared with SFMCs. Gene ontology analysis revealed 182 of these genes were involved in 13 different pathways. However, none of these pathways showed statistically significant dysregulation ($P < 0.05$). Among these genes, the highest number (36) had an immunological function (supplementary Table 2, available as supplementary data at Rheumatology Online). From this list we validated Granzyme H, killer cell lectin-like receptor subfamily F member 1 ($KLRF1$), natural killer group 7 ($NKG7$), killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail ($KIR3DL3$), cluster of differentiation (CD)1c, CD1d, CD244, CCR7, CD248 and Fas apoptotic inhibitory molecule 3 ($FAIM3$) genes by RT-PCR and soluble CD163 (sCD163) by ELISA.

Validation results

Comparison of normalized $C_t$ values showed no difference in gene expression between patient PBMCs and normal PBMCs, whereas, compared with PBMCs, patient SFMCs had a significantly different gene expression profile (Fig. 1). Compared with PBMCs, patient SFMCs had lower fold change in expression of Granzyme H, $KLRF1$, $NKG7$, $KIR3DL3$, CD244, CCR7, CD248 and FAIM3, whereas CD1c and CD1d were overexpressed (supplementary Fig. 2, available as supplementary data at Rheumatology Online).

ELISA for sCD163 was done for all samples included in microarray experiments, plus some additional stored samples. Thus we had 27 ERA plasma samples (median age at collection 16 years, 20 were on NSAIDS, 9 were on MTX, 1 female, 26 HLA-B27 positive), with 17 paired SF and 32 healthy plasma samples (median age 23 years, all male).

There was no significant difference between sCD163 levels (ng/ml) of ERA plasma [median 1080; interquartile range (IQR) 622.5–1992] and healthy plasma (median 786; IQR 430.6–1349). However, ERA SF (median 4425; IQR 3247–8046) had considerably higher sCD163 than plasma (median 1080; IQR 622.5–1992; $P < 0.001$; Fig. 2).

Discussion

To our knowledge, this is the first study showing that SFMCs of ERA patients have a gene expression profile...
distinct from their PBMCs. Many of these genes were of immunological relevance and SFMCs had higher expression of genes associated with antigen presentation, scavenger function, chemotaxis and proteases, whereas genes involved in NK cell function, cell adhesion and inhibitors of apoptosis were underexpressed. No significant difference was seen between the expression profiles of PMBCs from ERA patients and healthy control subjects.

The data on differential gene expression at the PBMC level vary between various studies. Barnes et al. [8] found that ERA PBMCs and healthy PBMC expression profiles differed from each other, with 193 genes and 2 pathways (tyrosine metabolism and axonal guidance signalling) different between them. However, no immune-related pathways were dysregulated in ERA. Although Barnes’ study comprised drug-naive patients with recent-onset disease, almost half of the patients had no active joints. In contrast, the present study had all patients with active disease and still there was no significant difference in PBMC expression profile between different groups. This suggests that the immune events in ERA are localized to the synovial compartment and very few changes are evident in PBMCs.

We found that SFMCs (compared with PBMCs) had downregulation of genes involved in NK cell function such as Granzyme H and KLRF1, and NKG7, which is expressed not only on NK cells, but also on cytotoxic T lymphocytes. CD244 (NK cell receptor 2B4) and KIR3DL3 are known to block NK cell function. Thus our findings suggest that NK cell cytolytic function is dysregulated in the synovial compartment. NK cell dysfunction has been reported in patients with SoJIA, especially in association with macrophage activation syndrome [10]. Although there are no data available on NK cell dysfunction in ERA, we still need to establish the extent to which downregulation of these genes interferes with NK cell function. The other reason could be fewer NK cells in the SFMC population compared with PBMCs.

Among genes upregulated in the SFMCs, there were genes associated with antigen presentation. CD1 is a family of glycoproteins involved in the presentation of lipid antigens to T and NKT cells, thereby influencing both adaptive and innate arms of the immune system. They can also present self-antigens, therefore they are implicated in autoimmune diseases like RA [11]. CD1 dysregulation has not been reported in JIA, and its involvement needs to be investigated further.

Although ERA has been classically associated with MHC class I antigens HLA-B*27 [12], involvement of MHC class II molecules with failure to attain remission and disease susceptibility has been shown [13, 14]. Recently it has also been shown that MHC class II molecules play an important role in innate immune responses, by modulating the response of Toll-like receptors (TLRs) to their cognate ligands [15]. The overexpression of MHC class II could also be related to the inflammatory milieu. The overexpression of classical and non-classical MHC molecules in the SFMCs may result in an exaggerated and detrimental immune response, either by increased antigen presentation or by aiding TLR-mediated inflammation.

ERA SFMCs had overexpression of chemokine genes such as CXCR3 and CXCL16. Similar to the present study, Barnes et al. [16] also found overexpression of several chemokines in SFMCs of all JIA subtypes. We have previously shown an increased frequency of CXCR3+ T cells in the SF of patients with ERA compared with PB [17]. Overexpression of chemokine receptors and their cognate ligands at the local site may contribute to increased migration of activated immune cells, thereby leading to increased inflammation.

Other genes of interest were CD163 and cathepsins. Soluble CD163 exerts anti-inflammatory effects and is increased in pathological conditions [18]. Our finding of increased mRNA of CD163 in SFMCs and sCD163 in SF suggests that it may play a role in the pathogenesis of ERA. Cathepsin B and L are members of the papain-like cysteine protease family and are important in the degradation of endocytosed and intracellular proteins. They are also involved in antigen presentation and bone resorption and activation of MMP [19]. Thus their overexpression in the SFMCs may suggest their role in bone and cartilage damage.

Even though we have not compared our data with SFMCs from a disease control, previous studies have shown overexpression of multiple chemokines and chemokine receptors similar to our data, suggesting that some of these changes may be related to inflammation [8, 20].

Our study has some limitations, including small sample size, heterogeneous population and the use of DMARDs by some patients. Secondly, comparison of the gene expression profile of ERA SFMCs with that of SFMCs from another inflammatory disease could have helped to determine whether the genes identified in our study were truly disease specific or merely hallmarks of inflammation. Also, we did not estimate the number of immune cells in PBMCs/SFMCs of our subjects before gene expression assays. Thus it is possible that difference in cell
populations may have contributed to observed differences in expression. Nevertheless, the present study does suggest that immune-related events resulting in inflammation are occurring predominantly in the synovial compartment in patients with ERA.

**Rheumatology key messages**

- Gene expression profiling suggests immune dysregulation in ERA SFMCs.
- ERA SFMCs overexpress genes involved with antigen presentation, chemokines and scavenger function.
- ERA SFMCs underexpress genes involved with NK cell function and apoptosis.

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