IL-1 and mevalonate kinase deficiency
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Cytokine and chemokine profiles in mevalonate kinase deficiency

Saskia H.L. Mandey, Janet Koster, Joost Frenkel, Hans R. Waterham
Cytokine and chemokine profiles in mevalonate kinase deficiency

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Abstract

Mevalonate kinase deficiency (MKD) is an autosomal recessive metabolic disorder characterized by recurring episodes of high fever and inflammation. The enzyme mevalonate kinase (MK) catalyzes the phosphorylation of mevalonic acid, which is an early step in isoprenoid biosynthesis. How a defect of isoprenoid biosynthesis contributes to (periodic) fever and inflammation remains unclear. In MKD patients both pro- and anti-inflammatory cytokines are elevated suggesting that a dysregulation of the cytokine network could play role in the pathogenesis of MKD. To gain further insight in this, we studied the effect of MKD on the expression of genes and proteins involved in the cytokine responses after ex vivo lipopolysaccharide (LPS) stimulation of peripheral blood mononuclear cells (PBMCs) from MKD patients and control subjects.

Cytokine protein expression analysis using cytokine antibody arrays showed that the secretion of interleukin (IL)-1β, IL-1β-dependent IL-6 and GRO (growth related oncogen) cytokines and, to a lesser extent, TNF-α is markedly higher in LPS-stimulated blood samples from the MKD patients than in those from the control subjects.

mRNA expression arrays indicated that the transcription of the genes encoding IL-1β, IL-1α, IL-6 and TNF-α is somewhat higher in LPS-stimulated PBMCs from the MKD patients than in those from control PBMCs. Our combined results suggest that the mechanism of inflammatory response after LPS stimulation does not differ much between MKD and control PBMCs, but that MKD cells show a stronger and faster response leading to higher levels of IL-1β and a more activated cytokine network.

Introduction

Recurrent episodes of high fever and inflammation are the most characteristic clinical features of mevalonate kinase deficiency (MKD). These episodes are often accompanied by headache, arthritis, nausea, abdominal pain, diarrhea, and skin rash (1;2). Originally, two distinct syndromes had been defined, classic mevalonic aciduria (MA) (3) and hyper-IgD and periodic fever syndrome (HIDS) (4), which are now known to represent the severe and mild presentation of MKD (5). Patients with the MA presentation show, in addition to the inflammatory episodes, developmental delay, dysmorphic features, ataxia, cerebellar atrophy, and psychomotor retardation and may die in early childhood (6).
During fever episodes, MKD patients show an acute phase response including leukocytosis, granulocytosis, increased erythrocyte sedimentation rate, high levels of serum amyloid A, C-reactive protein, fibrinogen, soluble type II phospholipase A$_2$ and α$_1$-glycoprotein (7). The levels of α$_1$-glycoprotein are also elevated between the fever episodes, indicating a persistent state of inflammation (8). The acute phase response during the fever episodes correlates well with the cytokine alterations that have been reported in previous studies. These revealed that during the fever episodes the circulating interleukin (IL)-6, IL-1ra, sTNFr p55, sTNFr p75 are elevated in MKD patients (9). In addition, ex vivo stimulation of peripheral blood mononuclear cells (PBMCs) from MKD patients with lipopolysaccharides (LPS) also resulted in elevated levels of IL-1β, TNF-α and IL-1 receptor antagonist (IL-1ra) (10;11). These data point to an activation of monocytes/macrophages in vivo.

MKD is caused by a deficient activity of the enzyme mevalonate kinase (MK), which catalyzes the phosphorylation of mevalonic acid, an early step in isoprenoid biosynthesis pathway. Isoprenoids function in a variety of biological processes such as cell proliferation and differentiation, glycosylation and intracellular signal transduction (12). At this moment it is not clear how a defect of isoprenoid biosynthesis contributes to (the onset of) recurrent fever and inflammation.

In this pilot study, we have compared the cytokine and chemokine profiles induced by LPS-stimulation of PBMCs from MKD patients with those from control subjects with the aim to obtain insight into possible mechanisms that may lead to a dysregulation of the cytokine response in MKD.

**Materials and Methods**

After approval by the ethical review board and obtaining written informed consent from the parents, blood was drawn from MKD patients by venipuncture in sterile pyrogen-free heparinized tubes (Vacuette, Greiner Bio-one). The patients included in this study all clinically present with the HIDS phenotype and were afebrile at the time of sampling. Healthy volunteers served as controls.

PBMCs were isolated by density gradient centrifugation using Lymphoprep™ according to the supplier’s protocol (Axis-Shield, Oslo, Norway). 2x10$^6$ PBMCs per well were seeded and cultured in 12 well-flat-bottom microtiter plates in RPMI 1640 (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 10 mM HEPES (Gibco) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO$_2$ in air. After 18 hours, *E. coli* 055:B5 lipopolysaccharide (LPS) (Sigma, final concentration of 200 ng/ml) or medium was added to the cultures followed by 4 hours of additional incubation. After this, supernatants were collected by centrifugation (10 minutes; 750 g) and stored at −80°C until analysis. The cell pellets were washed once with phosphate-buffered saline and twice with 0.9% NaCl, dissolved in TRIzol (Invitrogen, Carlsbad, CA) and stored at −80°C until analysis.

Fresh blood samples of patients and controls were diluted 1:3 in RPMI 1640 (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10 mM HEPES (Gibco) and 1% penicillin-streptomycin and cultured in 96 well-flat-bottom microtiter plates in a total volume of 100 µl. After 18 hours, *E. coli* 055:B5 LPS (Sigma, final concentration of
100 ng/ml) or medium was added to the whole blood cultures followed by 24 hours of additional incubation.

**Cytokine Measurements**

Antibody array analysis were performed using the Human Cytokine Antibody Array membranes C series 2000 (RayBiotech, Norcross, GA, USA) according to the manufacturer’s instructions. With these blots 174 different cytokines and chemokines can be detected (for detailed table, see addendum 1). Densitometric analysis of the immunoblots was performed using the Advanced Image Data Analyzer (AIDA) software (Raytest, Strauenhardt, Germany). IL-1β, IL-6, IL-10, IFN-γ and TNF-α were measured in duplicate in thawed supernatant and whole blood samples, using commercially available enzyme-linked immunosorbert assays (Pelikine-compact™ human IL-1β, IL-6, IL-10, IFN-γ and TNF-α ELISA kits, Sanquin, Amsterdam, The Netherlands) according to the manufacturer’s instructions.

**Quantitative real-time RT-PCR analysis**

The relative expression levels of IL-1β, GRO (growth related oncogen) and β-actin mRNAs were determined with the LightCycler System using the LightCycler FastStart DNA Master SYBR green I kit (Roche, Mannheim, Germany). To this end, total RNA (free of genomic DNA by using RNase-free DNase (Promega, Madison, WI, USA)) was isolated from PBMCs using the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA), after which first strand cDNA was prepared using the first strand cDNA synthesis kit for RT-PCR (AMV) according to the manufacturer’s instructions (Roche, Mannheim, Germany). The IL-1β cDNA fragment was amplified using primers: Fw 5’-AGAAGAACCTATCTTCTTCGAC-3’ and Rev 5’-ACTCTCCAGCTGAGTGGTGG-3’. GRO cDNA primers were: Fw 5’-TGCGCCTACACCC-3’ and Rev 5’-TGAGGATTGAGGCAAGCTT-3’. The β-actin cDNA fragment was amplified using the following primer set: Fw 5’-GGGACCAGGCGGTGATGG-3’ and Rev 5’-GTCACACATGATCGGTC-3’. Data were analyzed using LightCycler Software, version 3.5 (Roche, Mannheim, Germany) and the program LinRegPCR, version 7.5 (13) for analysis of real-time PCR data. To adjust for variations in the amount of input RNA, the levels of the IL-1β and GRO cDNAs were normalized against the levels of the housekeeping gene β-actin.

**mRNA expression analysis**

mRNA expression analysis was performed using the Oligo GEArrays Toll-Like Receptor Signaling Pathway (EHS-018.2) and Autoimmune and Inflammatory Response (OHS-803) from SuperArray Bioscience Corporation (Frederick, MD, USA) following the manufacturer’s protocol. In total, the two combined arrays include oligonucleotides that recognize 505 different transcripts of genes involved in these pathways and processes (for detailed gene tables, see addendum 2). Briefly, 500 ng total RNA isolated from PBMCs was reverse transcribed into Biotin-16-dUTP-labeled cRNA probes with the use of True Labeling-AMP™ 2.0 (SuperArray Bioscience) according to the manufacturer’s protocol. The Oligo GeArray membranes were prehybridized at 60 °C for at least 2 hours. Hybridization was carried out by
incubation of the membranes with Biotin-labeled cRNA (2µg) probes at 60 °C overnight. The hybridized membranes were washed twice in 2×SSC with 1% SDS and once in 0.1×SSC with 0.5% SDS, further incubated with alkaline phosphatase-conjugated streptavidin and finally developed with CDP-Star chemiluminescent substrate. Images were quantified using data analysis software (GEArray Expression Analysis Suite).

Results

Increased IL-1β levels in whole blood samples from MKD patients

We performed a cytokine antibody array with whole blood samples from two MKD patients and two controls after stimulation with LPS. Of the 174 different cytokines and chemokines that can be detected with this array (see addendum 1), in particular IL-1β levels were markedly higher in MKD patients when compared to controls. In addition, the secreted levels of GRO (also known as chemokine (CXCL) 1,2,3), IL-6, IL-10 and TNF-α were somewhat more increased in the MKD samples (not shown). These data suggest that the LPS response is stronger in MKD patients than in control subjects.

Figure 1: Cytokine levels in whole blood samples from two MKD patients and two control subjects cultured in the absence (open bars) and presence (closed bars) of LPS. The levels were determined by ELISA for IL-1β (A), IL-6 (B), IL-10 (C) and TNF-α (D).

To confirm and substantiate these observations, we performed ELISA analysis of IL-1β, IL-6, IL-10 and TNF-α using the same whole blood samples that were used in the
cytokine antibody array (figure 1). The increased cytokine levels could be confirmed for IL-1β, IL-6 and TNF-α. IL-10 production, however, was only increased in MKD patient 2. GRO expression was confirmed at the mRNA level, which showed that in MKD cells, GRO mRNA levels were increased two-fold in comparison to those in control cells (figure 2), which is line with the increased GRO levels observed with the cytokine antibody array.

![GRO mRNA expression](image)

**Figure 2:** Relative mRNA expression of GRO in PBMCs from two MKD patients and two control subjects, presented as GRO/β-actin ratio. The mRNA expression levels were determined with the LightCycler system.

**Small differences in cytokine gene expression levels**

In order to obtain possible insight into the molecular mechanisms that regulate the onset of the periodic fever and inflammation in MKD, we also studied the expression of genes involved in cytokine response in LPS-stimulated PBMCs using Oligo GEArrays (figure 3). MKD and control cells did not show much difference in the expression of most chemokines, including CC-chemokines and CXC-chemokines. The mRNA levels of the genes encoding the proinflammatory cytokines TNF-α and IFN-gamma were very low, but slightly higher in MKD than in controls. Although on the cytokine antibody array, IL-1α levels were barely detectable, the IL-1α mRNA was expressed both in MKD as in controls. The explanation for this is that IL-1α is not secreted from cells, but remains intracellular or is membrane bound and functions by cell contact or after cell lysis. Also IL-1β, IL-6 and TNF-α mRNAs were observed in both MKD and control PBMCs with levels somewhat higher in MKD PBMCs. Because the differences in IL-β mRNA levels between MKD and control PBMCs on the Oligo GEarray were very small in comparison to the marked difference in IL-1β protein levels in whole blood, we also measured IL-β mRNA expression with the LightCycler system using different oligonucleotide probes than used on the array. This showed a 3 to 4-fold increase in IL-1β mRNA levels in MKD PBMCs stimulated by LPS in comparison to control cells stimulated by LPS (figure 4), suggesting that the probes used on the oligo GEarray does not recognize all IL-1β mRNAs.

In the line with the observed mRNA levels, we measured in the supernatants of these PBMCs secreted IL-1β, IL-6, IL-10, IFN-γ and TNF-α (figure 5). IFN-γ was undetectable in supernatants of both controls and MKD patients.
Cytokine and chemokine profiles in MKD

Figure 3: Cytokine gene expression levels in PBMCs from controls and MKD patients after stimulation with LPS. Expression levels of the mRNAs for TNF-α (B), IFN-γ (C), IL-1α (D), IL-1β (E), IL-6 (F) were determined by densitometric analysis of the arrays.
Figure 4: Relative mRNA expression of IL-1β in LPS-stimulated PBMCs from two MKD patients and two control subjects, presented as IL-1β / β-actin ratio. The mRNA expression levels were determined with the Lightcycler system.

Figure 5: Cytokine levels secreted by PBMCs from two MKD patients and two control subjects, cultured in the absence (open bars) and presence (closed bars) of LPS. The levels were determined by ELISA for IL-1β (A), IL-6 (B), IL-10 (C) and TNF-α (D).
**Discussion**

Cytokines are regulators of host responses to infection and injury. Some cytokines act proinflammatory, whereas others serve to reduce inflammation (anti-inflammatory). IL-1β and TNF-α are central proinflammatory and pyrogenic (fever-causing) cytokines, which are able to activate additional inflammatory pathways, including pathways producing additional cytokines, via transcription factors such as NF-κB. In MKD, the cytokine network is continuously activated and this dysregulation could play a role in the pathogenesis of this disease. In this study we tried to obtain insight into the molecular mechanisms that regulate the onset of or predispose to periodic fever and inflammation by studying the effect of MKD on the expression of genes and proteins involved in the cytokine response. Using cytokine antibody arrays, we observed as the most striking difference between MKD and control whole blood culture samples a marked increased protein expression of IL-1β in MKD. High IL-1β levels are known to lead to increased IL-6 and GRO secretion, which was confirmed using ELISA for IL-6 and mRNA analysis for GRO. GRO is a proinflammatory cytokine the expression of which is regulated by IL-1β in part through message stabilization (14-16). It is a chemokine specialized in recruitment of monocytes, the cell type that is the major source of IL-1β. The elevated GRO levels could therefore be both the cause as well as the result of increased IL-1β secretion.

Expression arrays probed with cRNAs prepared from mRNAs isolated from LPS-stimulated PBMCs from MKD patients and control subjects indicated that the transcription of the genes encoding IL-1 beta, IL-6 and TNF-α is more elevated in the PBMCs from the MKD patients than in control PBMCs. However, overall there were no large differences observed between the expression levels of genes involved in inflammation in the LPS-stimulated PBMCs from MKD patients and control subjects included in this pilot study.

From our combined results it can be concluded that the mechanism of inflammatory response after stimulation with LPS does not differ between MKD and controls, but that MKD cells react stronger to a stimulus, leading to higher levels of secreted IL-1β and a more activated cytokine network. These results require confirmation in a larger group of MKD patients and control subjects.

IL-1β has been postulated to play an important role in the pathogenesis of several autoinflammatory diseases including MKD (17). This is supported by the beneficial effect of treating patients with these diseases using a recombinant form of IL-1 receptor antagonist, anakinra (18-21). Although anakinra is not successful in all MKD patients, IL-1β indeed also seems to play an important role in the pathogenesis of MKD.

The synthesis, processing and release of mature IL-1β are tightly regulated events (22) and multiple signaling pathways are involved in the transcriptional upregulation of proIL-1β, including pathways triggered by IL-1β itself, by other inflammatory cytokines such as TNF, or by various Toll-like receptor ligands such as LPS (23). The conversion of proIL-1β into the active mature 17 kDa IL-1β form requires the cleaving by the IL-1β converting enzyme (ICE, also known as caspase-1). In PBMCs and whole blood cultures of MKD patients the secreted IL-1β levels were 50-fold increased.
compared to controls and at the transcription level we found a 4-fold increase in MKD PBMCs compared to control PBMCs. This indicates that the increased IL-1β secretion by MKD PBMCs and in whole blood cultures are at least partly due to an increased transcription level. Studies towards the exact regulation of IL-1β secretion in MKD may therefore aid in identifying potential therapeutic targets.

Acknowledgments

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References

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Addendum 1: Human Cytokine Antibody Array membranes C series 2000
Addendum 2

Toll-like Receptor Signaling Pathway

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Cytokine and chemokine profiles in MKD
Addendum 3

Autoimmune and Inflammatory Response

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<tr>
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<td>Data G</td>
<td>Data H</td>
<td>Data I</td>
<td>Data J</td>
</tr>
<tr>
<td>Data K</td>
<td>Data L</td>
<td>Data M</td>
<td>Data N</td>
<td>Data O</td>
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