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Myeloid related proteins are up-regulated in autoimmune thyroid diseases and activate toll-like receptor 4 and pro-inflammatory cytokines in vitro

Shiqiao Peng[a,1], Xuren Sun[b,1], Xinyi Wang[c], Haoyu Wang[a], Zhongyan Shan[a], Weiping Teng[a,*,2], Chenyan Li[a,*,2]

[a] Department of Endocrinology and Metabolism, Institute of Endocrinology, Liaoning Provincial Key Laboratory of Endocrine Diseases, The First Affiliated Hospital of China Medical University, Shenyang, Liaoning 110001, PR China
[b] Department of Gastroenterology, The First Affiliated Hospital of China Medical University, Shenyang, Liaoning 110001, PR China
[c] Department of Laboratory Medicine, The First Affiliated Hospital of China Medical University, Shenyang, Liaoning 110001, PR China

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ABSTRACT

Purpose: Myeloid-related protein (MRP) family plays an important role in the promotion of cell proliferation and the production of inflammatory cytokines. We investigated the expression of MRP6, MRP8 and MRP14 in thyroid tissues, serum, and peripheral blood monocular cells (PBMCs) in patients with autoimmune thyroid diseases (AITD).

Method: The expression of MRP6, MRP8, and MRP14 was investigated using immunohistochemical staining and quantitative real-time polymerase chain reaction in the thyroid glands of 7 patients with Graves' disease (GD), 8 with Hashimoto's thyroiditis (HT), and 7 healthy controls (HC). The serum levels of MRP8/MRP14 complex and MRP6 were investigated in 30 patients with GD, 36 with HT, and 30 with HC. The mRNA expression of MRP proteins in PBMCs was also explored. PBMCs from each group were incubated with MPRs and their effect on Toll-like receptor 4(TLR4) expression and their effect on the levels of the pro-inflammatory cytokines in supernatant were analyzed upon incubating with TLR4 and signaling pathways inhibitors.

Results: Serum levels of MRP8/MRP14 and MRP6 were up-regulated in patients with AITD. In addition, mRNA expression of MRP proteins in PBMCs and the thyroid gland was markedly elevated in AITD patients. MRP6 and MPR8 promoted the secretion of TNF-α and IL-6 in cultured PBMCs, and this elevation was more pronounced in AITD patients; we also found that this up-regulation was regulated by TLR4/phosphoinositide 3-kinase/nuclear factor-κB signaling pathway.

Conclusion: The expression of MRP proteins was elevated in AITD patients. Therefore, an MRP-TLR4 dependent signaling may play an important role in the pathogenesis of AITD.

1. Introduction

Innate immunity plays a vital part in the development of inflammation. The neutrophils and monocytes that form a crucial aspect of the immune system can sense injured cells and initiate adaptive innate immune response. These cells express pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) and also recognize danger-associated molecular patterns (DAMPs) secreted by damaged tissues and, as in the case of myeloid-related protein (MRP) family proteins, dying cells [1]. MPR proteins can be used as vital inflammatory regulators of immune system [2].

The MPR protein family features 21 protein sub-types, which are highly homologous and exert pleiotropic effects on small-molecular-weight calcium-binding proteins, acting on intracellular enzymes, cytoskeletal proteins, receptors and transcription factors [3]. All MRP proteins exert biological activity through shared structural motifs of the EF-hand domain [4]. These proteins play an important role in the promotion of cell proliferation and differentiation, apoptosis, and the production of inflammatory cytokines. MPR proteins also exert a vital effect on cell migration and energy metabolism, particularly in the maintenance of calcium homeostasis [3]. The present study involved three specific MRP proteins:MRP6(calgranulin C), MRP8(calgranulin A), and MRP14(calgranulin B), which are associated with the innate immune response via their expression on myeloid cells. MRP8, MRP14,

* Corresponding authors.
E-mail addresses: twp@vip.163.com (W. Teng), chenyan830424@126.com (C. Li).
[1] Shiqiao Peng and Xuren Sun contributed equally to this work.
[2] Chenyan Li and Weiping Teng contributed equally to this work.

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and MRP6 are also referred to as calcium binding proteins (S100A8, S100A9, and S100A12, respectively) [5]. Of these, MRP8 and MRP14 both form a stable homodimer (also known as calprotectin) in a calcium-dependent manner. The MRP8/MRP14 complex is expressed mostly in myeloid lineage cells, such as neutrophils, monocytes, activated macrophages, and the increased expression of these complexes has been demonstrated in a range of inflammatory diseases [2]. MPR6 is mainly expressed by neutrophils, with only low levels of expression in lymphocytes and nuclear cells [6]. Previous research has shown that MRP6 plays a role in the mediation of multiple cellular activities, including the inflammatory response. In fact, MPR8, MRP9, and MRP6 are highly expressed in a range of autoimmune diseases, such as rheumatoid arthritis (RA) [7], systemic lupus erythematosus (SLE) [8,9] and inflammatory bowel disease (IBD) [2]. There is a significant association between MRP proteins and disease activity in patients with both RA [9] and SLE [8,9]. However, the pathogenesis involved is complex, and involves intracellular and extracellular elements in the modulation of calcium signaling, and the inflammatory response of leukocytes in response to the activation of immune-related signaling.

Toll-like receptor 4 (TLR4) and the receptor for advanced glycation end products (RAGE) have both been implicated as receptors for MRP8/MRP14complexes, as well as MRP6. The MRP8/MRP14complex activates myeloid differentiation factor 88 (MyD88)-TLR4 signaling; triggers activation of the nuclear factor (NF)-κB pathway; and induces the release of pro-inflammatory cytokines including TNF-α and IL-17 [10,11]. MRP8 and MRP14 also interact with RAGE, causing activation of NF-κB signaling which accelerates the secretion of cytokines. Binding to RAGE generates the secretion of pro-inflammatory cytokines which may activate MRP6 in monocytes and thus up-regulate the production of MRP6on neutrophils [2].

Autoimmune thyroid disease (AITD) is an organ-specific autoimmune disease, predominantly including Hashimoto's thyroiditis (HT) and Graves' disease (GD). It affects almost 10% of the population [12]. HT is characterized by lymphocyte cellular infiltration and high serum levels of thyroperoxidase antibodies (TPOAb) and antithyroglobulin antibodies (TgAb). The thyroid-stimulating hormone receptor (TSHR) is the most important auto-antigen in GD. It has been established that the development of AITD is associated with a combination of genetic, environmental, and immune factors. Of all the environmental factors, exposure to iodine is one of the most important causes of AITD activation [13], although the specific mechanisms underlying AITD remain unknown.

Although MRP proteins have been investigated in a range of autoimmune diseases, the exact mechanism underlying AITD requires further elucidation. In the present study, we aimed to compare the expression of MRP proteins, including the MRP8/MRP14 complex and MRP6, in HT and GD patients and in healthy subjects. MRP protein expression was assessed in the thyroid, peripheral blood mononuclear cells (PBMC) and in serum from AITD patients. We also investigated the correlation between MRP proteins and disease activity. In addition, we used human PBMCs from AITD patients and controls to study the in vitro ability of MRP6, MRP8, MRP14, and the MRP8/MRP14 heterodimer to promote the production of pro-inflammatory cytokines. Finally, we used a specific TLR4 and signaling pathways antagonist to block TLR4 signaling, in order to determine the effect of MRP protein on PBMCs in a TLR4-dependent manner. Collectively, our data indicated that MRPs represent a promising therapeutic option for inhibiting inflammatory response.

2. Materials and methods

2.1. Study participants

Five ml of fresh blood was collected from 36 patients with HT, 30 patients with GD, and 30 age- and gender-matched healthy subjects. PBMCs from each group were extracted for further study and serum samples were collected; all samples were stored until required for analysis. Subjects from each group were gathered from the First Affiliated Hospital of China Medical University. All participants provided an entire history of physical examination. None of the patients were using levothyroxine or other anti-thyroid drugs. Participants with diabetes, or any other chronic disease, infectious disease, or cancer, were excluded from our study group. Levels of serum thyrotropin (TSH), free T3 (FT3), free T4 (FT4), TPOAb, TgAb, and thyrotrophin receptor antibody (TRAb) were analyzed using electrochemiluminescent immunoassays with an Cobas Elesys 601 system (Roche Diagnostics Ltd., Switzerland). All data were demonstrated in Table 1.

Table 1
The clinical characteristics of patients with Hashimoto's thyroiditis and Graves' disease and healthy controls.

Variable	HC	HT	GD	Normal range
No.	30	36	30	–
Age (years)	37 ± 8	34 ± 9	30 ± 9	–
Gender (M/F)	2/28	4/32	4/26	–
TSH (mIU/L)	1.5 ± 0.2	13.2 ± 2.64	0.007 ± 0.01	0.35–4.94
FT4 (ng/dL)	17.3 ± 1.9	12.9 ± 2.2	47.09 ± 13.7	9.01–19.05
FT3 (pg/mL)	4.2 ± 0.4	3.7 ± 0.2	32.3 ± 7.9	2.63–5.70
TRAb (IU/L)	–	–	49.6 ± 11.7	0–1
TPOAb (IU/mL)	3.53 ± 0.6	336.35 ± 27.7	123.5 ± 17.2	0.11–5.23
TgAb (IU/mL)	2.87 ± 0.4	407.96 ± 204.2	157.34 ± 15.97	0.81–3.83

Data are expressed as mean ± standard deviation according to the distribution. M, male; F, female. "-" represents that the experiment wasn't performed, or the data isn't applicable. HT, Hashimoto's thyroiditis; GD, Graves' disease; HC, healthy controls.

Thyroid tissue was collected from seven patients with untreated GD and eight patients with HT. In addition, seven normal thyroids were obtained from patients with benign thyroid goiter and undergoing surgery. All samples were obtained during scheduled thyroid operations and were stored at −80 °C to await further analysis.

2.2. Analysis of cytokine levels

Serum levels of MRP8/MRP14 were analyzed by quantitative ELISA (HyCult Biotechnology, Uden, Netherlands) in accordance with the manufacturer's specifications. Plasma MRP6 levels were also analyzed by ELISA (Cusabio, Wuhan, China). Absorbance was examined at a wavelength of 450 nm using a microplate reader which assessed optical density in 96 wells. PBMCs from each group were cultured in 24 well plates containing PRMI1640 (Hyclone, USA) with 10% fetal calf serum, 100 IU/ml of penicillin, and l00 μg/ml of streptomycin. PBMCs were primed with 5 μg/ml recombinant MRP6, MRP8, MRP14 monomer, and MRP8/MRP14 complex for 72 h, or incubated with no stimulation as a basal control. Supernatants were collected and stored at −80 °C in order to determine the levels of TNF-α and IL-6 by ELISA (eBioscience, San Diego, CA, USA) as evidence of pro-inflammatory cytokine activity. Subsequent experiment used a specific TLR4 antagonist, TAK-242, at a concentration of 1 μM (Invivogen, USA) to block TLR4 signaling after stimulation with MRP6 or MRP8. TNF-α and IL-6 were analyzed as described above.

2.3. Isolation of peripheral blood mononuclear cells

PBMCs were obtained from freshly collected blood in heparinized tubes, isolated by Ficoll-isopaque density gradient centrifugation (Gibco BRL, Life Technologies Ltd., Paisley, U.K.) as described previously [14] and washed free of platelets and Ficoll. Finally, Cells were mixed with 1 ml Trizol (Thermo Fisher scientific Inc., Waltham, MA, USA) and stored at −80 °C to await analysis.

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