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Increased expression of proprotein convertase enzyme FURIN in Rheumatoid Arthritis

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Abstract

Objective

FURIN is a proprotein convertase enzyme that inhibits the pro-inflammatory function of T cells and myeloid cells. Previously, elevated FURIN expression levels have been reported in immune-mediated diseases, such as primary Sjögren's syndrome. Here, we investigated the levels of FURIN in peripheral blood (PB) and synovial fluid (SF) leukocytes from patients with rheumatoid arthritis (RA).

Methods

FURIN mRNA expression in PB and SF cells was determined by qRT-PCR and FURIN plasma levels were measured using ELISA. Associations between FURIN levels and demographic and clinical characteristics of the patients were determined.

Results

FURIN levels were significantly elevated in PB and SF mononuclear cells, T cells and monocytes from RA patients when compared to healthy controls. High FURIN levels were significantly associated with the prevailing prednisolone treatment, higher prednisolone doses as well as increased CRP levels and Health Assessment Questionnaire (HAQ) values.

Conclusion

FURIN is significantly upregulated in RA PB and SF leukocytes, suggesting that FURIN may have a role in the pathogenesis of RA. In addition, our results suggest that elevated FURIN expression is associated with the indicators of more severe RA.

Keywords: RA, FURIN, proprotein convertase, biomarker, T cell, monocyte

Introduction

In RA, T cells, B cells, macrophages and dendritic cells accumulate into the joints. Different cell types promote inflammation by secreting pro-inflammatory cytokines, such as TNF-α, IFN-γ, GM-CSF, IL-1, IL-6 and IL-17 [1]. These events ultimately lead to chronic inflammation, synovial hyperplasia and degradation of articular cartilage and bone [2].

FURIN is the first discovered mammalian proprotein convertase (PCSK) enzyme. FURIN belongs to the subtilisin superfamily of serine endoproteases and it converts immature pro-proteins into functional units [3]. FURIN is essential for mammalian development and tissue homeostasis, but it also contributes to the pathogenesis of several diseases including cancers, elevated blood pressure and atherosclerosis [4][5][6].

FURIN is important in the regulation of the immune system. Deleting FURIN in mouse T cells results in a loss of peripheral immune tolerance and aberrant T helper cell polarization [7]. In the myeloid cells FURIN inhibits inflammatory responses by reducing the production of pro-inflammatory cytokines [8]. The function of FURIN in immunoregulation is not entirely clear, but its expression in immune cells is critical for the functional maturation of anti-inflammatory pro-TGF-β1 cytokine and the suppressive function of regulatory T cells [7].

Preliminary evidence also suggests that FURIN plays a role in the pathogenesis of human autoimmune diseases. In primary Sjögren's syndrome, an upregulated expression of FURIN has been detected in salivary gland biopsies [9], and in peripheral circulation [10]. Previously, we found an association between high plasma level of FURIN and a history of rheumatic disease in a prospective cohort study of patients with a suspected infection in the emergency room [11]. We have therefore investigated FURIN levels in peripheral blood (PB) and synovial fluid (SF) leukocytes from RA patients. We also explored possible associations between FURIN levels and demographic and clinical characteristics of the patients.

Patients and methods

Patient samples

PB and SF samples were collected from patients with active RA. The characteristics of the patient cohorts are presented in Tables 1 and 2. In addition, purified T cells and monocytes were obtained from eight RA patients: median (range) age 62 (37-87) years, duration of disease 18.5 (0.25-49) years, CRP 25 (18-67.4) g/L and ESR 35.5 (10-91) mm/h. Control samples were obtained from healthy blood donors (Finnish Red Cross Blood Transfusion Service, Tampere).

All patients gave their written informed consent. This study was approved by the Ethical Committee of Tampere University Hospital, Tampere, Finland, and conducted according to the principles of the Declaration of Helsinki.

Cell preparations

PB mononuclear cells (PBMCs) and SF mononuclear cells (SFMCs) were isolated by Ficoll-Paque Plus (Amersham Biosciences, Buckinghamshire, UK) density gradient centrifugation. T cells and monocytes were further purified from eight RA patients using magnetic beads (Miltenyi Biotec, Auburn, CA, USA). T cells were purified by negative selection (Pan T Cell Isolation Kit; Miltenyi Biotec) and monocytes were isolated by positive selection using anti-CD14-coated microbeads.

RNA isolation and quantitative RT-PCR analysis

Total RNA was isolated using the RNeasy MiniKit (Qiagen, Valencia, CA, USA). Total RNA was reverse transcribed using Maxima Reverse Transcriptase (Thermo Scientific, Waltham, MA, USA) and random hexamers (Thermo Scientific). The real-time PCR were done with the CFX96 instrument (Bio-Rad, Hercules, CA, USA) using Maxima SYBR Green/ROX master mix (Thermo Scientific).

The following primers for FURIN and TATA-binding protein (TBP, housekeeping) were used: 5′-GAATATAATCCCAAGCGGTTTG-3′ and 5′-ACTTCACATCACAGCTCCCC-3' for TBP and 5’-GGCAAAGCGACGGACTAAAC-3’ and 5’-CGTCCAGAATGGAGACCACA-3’ for FURIN.

Mean FURIN expression values from triplicate samples were obtained by dividing them by the mean values obtained for the TBP housekeeping gene.

FURIN ELISA

FURIN plasma levels were measured using the Human Furin ELISA kit (Thermo Scientific). Duplicate determinations were carried out for each sample. The analysis was performed with a cut-off by dividing samples into those that were below detection threshold (< 123 pg/ml) and above it (≥ 123 pg/ml)

Statistical analysis

Statistical analyses were performed using SPSS Statistics (IBM, version 22). Fisher´s Exact Test was used to test the relationship between two binomial variables. Analyses including ordinal and scale variables were conducted using Mann-Whitney U-Test (Exact). The correlations for continuous variables were calculated using Spearman´s correlation coefficient.

Results

Increased expression of FURIN in PB and SF T cells and monocytes from RA patients

First, we examined the levels of FURIN mRNA in PBMCs from patients with active RA and healthy controls. The expression of FURIN was significantly elevated in PBMCs from RA patients when compared with healthy controls (Figure 1A; $p<0.001$).

To gain better insight into the cell population which express the increased levels of FURIN, we studied the expression of FURIN mRNA in isolated PB T cells and monocytes. As shown in Figure 1B and 1C, the levels of FURIN were elevated in both PB T cells and monocytes from RA patients when compared to healthy controls, with monocytes expressing higher levels of FURIN than T cells.

In RA, synovial T cells and macrophages are exposed to various inflammatory stimuli, and show activated phenotype compared to the cells in PB. To investigate whether the cells from the inflamed joints express even higher levels of FURIN, SFMC, SF T cells and SF monocytes were isolated from patients with active RA presenting with arthritis of the knee joint. The levels of FURIN mRNA in SFMCs, T cells and monocytes were comparable to those in RA PB cells (Figure 1A-C).

Finally, we also evaluated FURIN plasma levels in patients with RA and healthy controls. Plasma FURIN levels in both healthy controls and patients with RA varied considerably and no significant difference was observed between RA patients and healthy controls (Figure 1D; $p=0.23$). However, more patients (10 out of 17, 59%) than controls (5 out of 14, 35%) demonstrated detectable levels of FURIN ($\geq$ 123 pg/ml).

Correlation of FURIN levels with clinical characteristics of RA patients

We next examined whether FURIN mRNA levels in PBMC or SFMC correlated with any of the demographic or clinical characteristics of RA patients. We found a significant correlation between current prednisolone use ($p=0.020$) and dose ($p=0.004$) and FURIN mRNA levels in PBMC. The expression of FURIN in SFMC, in turn, showed very strong correlation to the CRP level (Table 1; $p<0.001$). In addition, there were trends towards associations between

FURIN level in SFMC and ESR level (r=0.6667, p=0.0710) and prednisolone dose (r=0.6747, p=0.0664).

We also examined whether FURIN levels in plasma associated with any of the clinical characteristics. HAQ disability index (p=0.003) and prednisolone dose (p=0.023) were significantly higher in the group demonstrating detectable levels of FURIN (Table 2). In addition, IL-1β, IL-2, IL-4, IL-6, IL-7, IL-10, IFN-γ and TNF-α levels in plasma were determined in ten patients and their levels were correlated to those of FURIN. The median levels of all cytokines studied were higher in the group demonstrating detectable levels of FURIN (n=7) compared to patients presenting with undetectable FURIN levels (n=3; data not shown). However, this difference was significant only in the case of IL-2 (median 28 pg/ml versus 6 pg/ml, p=0.033).

Discussion

The expression of FURIN in RA has not been systematically studied earlier, and no data are available regarding FURIN expression in leukocytes from RA patients. Lin et al [12] reported upregulated expression of FURIN in synovial pannus derived from three patients with RA. In addition to RA, FURIN has been found to be upregulated in PBMCs and salivary gland biopsies in primary Sjögren's syndrome [9][10]. Elevated FURIN levels have also been seen in patients with systemic lupus erythematosus (SLE) [13]. Together, these previous results and our current findings showing increased FURIN expression in RA indicate that FURIN is upregulated in different types of rheumatic diseases.

Our results show that FURIN levels associate with prednisolone treatment and dose, CRP and ESR levels and HAQ disability index. Since prednisolone is used in the treatment of active RA, these findings suggest that FURIN is elevated mostly in patients with active and severe disease. This conclusion is supported by the fact that patients with elevated FURIN levels showed also global upregulation of plasma cytokines, particularly T-cell-activation-associated IL-2. These findings are also in line with our previous results indicating that FURIN expression is upregulated upon the activation of T cells and macrophages [5][7]. However, the inflammatory response in RA joints per se does not seem to result in further FURIN upregulation, since comparable levels of FURIN were observed in PB and SF samples.

Previous results suggest that FURIN has chiefly a protective role in arthritis. Systemic administration of FURIN was shown to ameliorate collagen-induced arthritis in mice [12]. In addition, inhibition of FURIN was shown to enhance the invasive phenotype of synoviocytes from patients with RA [14]. The protective role of FURIN in autoimmunity is also supported by previous results showing that FURIN is essential in maintaining peripheral immune tolerance [7] and it inhibits proinflammatory cytokine production in myeloid cells [8]. Notably, FURIN has also been reported to inhibit matrix metalloproteinase 13 and to limit osteoarthritis in mice [15]. The idea of using FURIN, its biologically active derivatives or its inhibitors in the treatment inflammatory diseases has been documented in patent applications (US20040127396A1, WO2011144517A1).

Our current results suggesting that FURIN expression associates with more active and severe RA, are in line with previous data showing the upregulation of anti-inflammatory cytokines

IL-10 and TGF-β [1] in RA patients. The overexpression of these regulatory proteins may reflect an attempt to combat inflammation, but due to the plethora of pro-inflammatory mediators present in RA, inflammation persists despite the actions of FURIN and other immunosuppressive proteins.

Taken together, our data demonstrate that FURIN is upregulated in severe RA. Further investigations are warranted to better determine the role of FURIN in the pathogenesis of RA and its potential value as a biomarker in inflammatory diseases.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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Table 1. The associations of patients' characteristics and clinical parameters with FURIN mRNA levels in PBMC and SFMC.

	FURIN mRNA in PBMC			**FURIN mRNA in SFMC**		
	N = 16	r	p-value	N = 8	r	p-value
Characteristic						
Age[a] (years)	66 (32 - 88)	0.0074	0.9784	54 (32 – 72)	– 0.0238	0.9554
Gender (male)	7 (44 %)		0.7577	6 (75 %)		0.8571
Duration of disease (years)	15 (0 - 37)	- 0.0517	0.8493	11 (1 - 37)	– 0.4762	0.2329
Clinical parameters						
CRP[b], g/l	30 (11 - 114)	0.3944	0.1306	44 (15 - 114)	0.9523	0.0003
ESR[c], mm/h	42 (10 - 118)	0.0383	0.8881	32 (10 - 88)	0.6667	0.0710
Number of swollen joints	7 (1 - 16)	0.3208	0.2258	7 (1 - 9)	0.3879	0.3423
Number of tender joints	6 (0 - 16)	0.2316	0.3880	3 (1 - 9)	– 0.1473	0.7278
DAS28[d] (ESR)	5.3 (3.0 - 6.3)	0.2235	0.4053	4.8 (3.0 - 5.6)	0.4192	0.3013
VAS[e], global health	56 (0 - 100)	0.4341	0.0929	47 (1 - 90)	0.5714	0.1390
VAS, pain	58 (0 - 100)	0.3765	0.1506	47 (2 - 92)	0.5714	0.1390
HAQ[f]	1.51 (0.00 - 2.50)	0.1550	0.5666	1.19 (0.00 - 1.74)	– 0.2892	0.4873
Rheumatoid factor positivity	10 (63 %)		0.1471	3 (38 %)		0.1429
Current treatment						
Prednisolon	12 (75 %)		0.0198	6 (75 %)		0.4286
Prednisolon dose (mg)	6.3 (0.0 - 15.0)	0.6809	0.0037	8.8 (0.0 - 25.0)	0.6747	0.0664

Methotrexate	7 (44 %)	0.6065	4 (50 %)	0.3429
DMARD[g]	15 (94 %)	0.2500	7 (88 %)	1.0000
Biologics	2 (13 %)	0.2667	4 (50 %)	0.8857

The data are reported as median (and range) or number (and percentage).

The expression of FURIN mRNA was calculated in relation to the TBP expression (FURIN/TBP)

[a]Age at the time of sampling.

[b]C-reactive protein.

[c]Erythrocyte sedimentation rate.

[d]Disease Activity Score.

[e]Visual Analogue Scale.

[f]Health Assessment Questionnaire.

[g]Disease-modifying antirheumatic drug.

Table 2. Patients' characteristics and clinical parameters in all plasma samples and in two groups showing FURIN plasma levels below or above the detection threshold of FURIN ELISA.

	All samples (N=17)	FURIN < 123 pg/ml (N=7)	FURIN ≥ 123 pg/ml (N = 10)	p-value
Characteristic				
Age[a] (years)	66 (52 - 88)	66 (58 - 77)	68 (52 - 88)	0.9056
Gender (male)	4 (24 %)	1 (14 %)	3 (30 %)	0.6029
Duration of disease (years)	7 (0 - 29)	7 (0 - 26)	8.5 (0 - 29)	0.9619
Clinical parameters				
CRP[b], g/l	18 (0 - 68)	16 (0 - 39)	20 (0 - 68)	0.3772
ESR[c], mm/h	40 (7 - 118)	40 (7 - 80)	41 (8 - 118)	0.5843
Number of swollen joints	8 (1 - 15)	7 (1 - 9)	8 (4 - 15)	0.1633
Number of tender joints	6 (0 - 37)	6 (3 - 25)	5.5 (0 - 37)	0.9423
DAS28[d] (ESR)	5.6 (3.5 - 6.9)	5.5 (3.7 - 5.9)	5.7 (3.5 - 6.9)	0.2698
VAS[e], global health	63 (0 - 93)	56 (0 - 85)	72 (5 - 93)	0.3638
VAS, pain	55 (0 -100)	54 (0 - 88)	64 (6 - 100)	0.5537
HAQ[f]	1.25 (0.63 - 2.75)	0.75 (0.63 - 1.63)	1.74 (0.75 - 2.75)	0.0031
Rheumatoid factor positivity	10 (59 %)	3 (43 %)	7 (70 %)	0.3500
Current treatment				
Prednisolon	13 (77 %)	4 (57 %)	9 (90 %)	0.2500
Prednisolon dose (mg)	7.5 (0.0 - 27.5)	5 (0.0 - 7.5)	10 (0.0 - 27.5)	0.0234

Methotrexate	8 (47 %)	3 (43 %)	5 (50 %)	1.0000
DMARD[g]	12 (71%)	5 (71 %)	7 (70 %)	1.0000
Biologics	4 (24 %)	1 (14 %)	3 (30 %)	0.6029

The data are reported as median (and range) or number (and percentage).

P-values were derived from comparisons of the groups showing FURIN levels below or above the detection threshold of FURIN ELISA

[a]Age at the time of sampling.

[b]C-reactive protein.

[c]Erythrocyte sedimentation rate.

[d]Disease Activity Score.

[e]Visual Analogue Scale.

[f]Health Assessment Questionnaire.

[g]Disease-modifying antirheumatic drug.

Figure legends

Fig 1: FURIN mRNA expression (A-C) and FURIN plasma levels (D) in patients with RA and healthy controls.

Levels of FURIN mRNA were analyzed by qRT-PCR in PB mononuclear cells (n=16) and SF mononuclear cells (n=8; A), T cells (n=7 for PB and 4 for SF; B) and monocytes (n=6 for PB and 4 for SF; C) from healthy controls and patients with RA. Paired PB/SF samples were available from three patients in the mononuclear cell analysis, from four patients in the T cell analysis and from three patients in the monocyte analysis. The data are presented as relative FURIN expression divided by TBP level (A-C). FURIN protein levels were measured by ELISA in plasma samples from healthy controls and RA patients (D). Horizontal lines indicate the mean expression levels in each group. The detection limit of the assay was 123 pg/ml. Significant differences between study groups are marked with an asterisk: *$p<0.05$, **$p<0.01$, ***$p<0.001$. RA: rheumatoid arthritis; PB: peripheral blood; SF: synovial fluid; TBP: TATA-binding protein.