

ORIGINAL ARTICLE

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Do cytokines play role in the pathogenesis of mucopolysaccharidosis

¹Asli Inci¹, ¹Asburce Olgac¹, ¹Canan Demirtas², ¹Ilyas Okur¹, ¹Gursel Biberoglu¹,
¹Fatih Suheyl Ezgu¹, ¹Leyla Tumer¹

¹Gazi University School of Medicine, Division of Pediatric Metabolism and Nutrition, Ankara, Turkey²Gazi University School of Medicine, Department of Medical Biochemistry, Ankara, Turkey

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Abstract

The mucopolysaccharidoses (MPS) are a heterogeneous group of inborn errors of metabolism with an increased deposition of glycosaminoglycans in lysosomes (GAGs). In MPS, GAG leads to inflammatory pathway activation. In MPS models, studies have shown that accumulation of glycosaminoglycans in lysosomes causes activation of oxidative stress, and then apoptosis is triggered. In this study, the aim was to show whether GAG deposition could trigger the inflammatory processes via cytokines. Forty-three MPS patients as patient group and 29 healthy children as control group were included in the study. Samples were taken before and after ERT from 8 MPS patients. 15 patients were treated with enzyme replacement and 28 patients could not be treated with the enzyme. Tumor necrosis alpha (TNF- α), interleukin 1-beta (IL-1 β), interleukin 6 (IL-6) levels were studied by ELISA method. When cytokine levels of MPS patients and control groups were compared, cytokines were found to significantly increased ($p \leq 0.05$) in MPS patients. Pretreatment IL-6 and posttreatment IL-6 and IL-1 β levels of patients treated with enzyme replacement therapy (ERT) were found near to control group. Pre-treatment and post-treatment TNF- α levels were found significantly higher in MPS patients than the control group that was significantly higher in the post-treatment group. In the MPS group of ERT treatment, IL-1 β and TNF- α levels decreased significantly after ERT ($p \leq 0.05$). MPS patients had higher levels of IL-6 and TNF- α levels than the control group that revealed inflammatory pathway activation in MPS patients. The inflammatory process is prominent in MPS patients that GAG deposition leads to increase pro-inflammatory cytokines and oxidative stress. Although enzyme replacement therapy reduces glycosaminoglycan accumulation by preventing cytokine production, it might be more effective when given with antiinflammatory mediators.

Keywords: Aupopolysaccharidosis, inflammation, cytokines, tumor necrosis alpha, interleukin 1 beta, interleukin 6

Introduction

Mucopolysaccharidosis (MPS) is a group of lysosomal storage diseases caused by deficiencies in enzymes in the catabolism of glycosaminoglycans (GAG) [1,2]. Excessive accumulation of unmetabolized GAGs leads to intralysosomal GAG deposition and then progressive damage of cells and tissues [2-4].

MPS can be divided into MPS I, II, III, IV, VI, VII, and IX. The clinical phenotype is characterized by somatic manifestations with respiratory and cardiovascular dysfunction, hepatosplenomegaly, corneal clouding, hearing dysfunction, skeletal deformities, and neurologic manifestations with learning difficulties, mental retardation, aggressiveness, and hyperactivity [5-8].

Inflammation is one of the mechanisms in the pathogenesis of MPS. GAG deposition initiates an inflammatory response through activation of some pathways especially the toll-like receptor -4 (TLR-4) pathway and some oxidative damages through the reactive oxygen species. Unfortunately, inflammatory response progressively increases in parallel with the GAG deposition in lysosomes [9]. Simonaro et al recently showed that GAG deposition induced some inflammatory events through the activation of the TLR-4 signaling pathway leading to apoptosis in cartilages and hyperplasia in synovial membranes [10-12]. TLR-4 signaling is thought to be induced by either LPS, classical pathway, or by the breakdown of extracellular matrixes [13-16]. Simanora et al also suggested that TLR-4 pathway activation leads to proinflammatory process activation via some molecules. Tumor necrosis factor-alpha (TNF- α) was suggested as the major cytokine playing a major role in MPS pathology and found in increased levels in the circulation and within the tissues of MPS animal models [12].

Nowadays, the enzyme replacement therapy (ERT) is available for

*Corresponding Author: Asli Inci, Gazi University School of Medicine, Division of Pediatric Metabolism and Nutrition, Ankara, Turkey
E-mail: aslidinci@gmail.com

five MPS disorders (MPS I, II, IV-A, VI, VII). ERT is effective by preventing the accumulation of undegraded substrates, glycosaminoglycans if ERT is initiated as early as possible, some clinical improvements have been observed in patients especially [17-19].

Considering elevated cytokine levels due to GAG deposition in animal models of MPSs, this study aimed to evaluate inflammatory cytokine levels primarily TNF- α , interleukin 1 beta (IL-1 β), and interleukin 6 (IL-6) in MPS patients and enzyme replacement therapy effect on cytokines levels in MPS patients.

Materials and Methods

Participants

MPS patients as patient group and healthy volunteers as control group were evaluated for the effect of cytokines. For that reason, blood samples of volunteers were obtained after informed consent was taken from families and patients. Forty-three MPS patients and 29 healthy volunteers were included in the study. Fifteen patients (MPS I, II, IV-A, VI and VII) were given ERT. Blood samples from 15 MPS patients were taken before and after enzyme replacement therapy. Twenty-eight patients could not be treated with ERT; 27 patients were MPS III and one patient was MPS II with severe mental retardation that enzyme replacement therapy could not be started. Diagnosis of MPS was confirmed either by specific enzyme levels or molecular analysis. Exclusion criteria were infection in any part of the body and concomitant other chronic diseases that could cause increased levels of cytokines in both MPS patients and the control group. Objects with infection and/or other chronic diseases were not included in the study. MPS types was shown in Table 1.

Sample collection and preparation

Blood samples were obtained from patients either immediately before the session of ERT and 4 hours after ERT in MPS patients treated with ERT or any time from non-ERT treated MPS patients or the control group. Samples were centrifuged for 20 min at the speed of 2000-3000 r.p.m and supernatant were removed then serums were stored at -80 °C until analysis.

Plasmatic proinflammatory cytokines (TNF α , IL-1 β , IL-6)

Plasma TNF α , IL-1 β , IL-6 were measured by commercial kit Human ELISA IL-6, IL-1 β , and TNF- α PicoKine (Boster/USA). The results were expressed as pg/mL.

Statistical analysis

Statistical analyses were performed with IBM SPSS 23.0. While

evaluating the study data, Chi-Square (c2) statistical method was chosen to compare descriptive statistical methods. The suitability of the data was evaluated by Kolmogorov-Smirnow and Shapiro-Wilk tests for the normality. In the study, Independent Samples t-Test to compare the normal distribution data between groups, and for non-normally distributed data in intergroup comparison Mann-Whitney U test were used. The paired samples t-test for normally distributed datas was the statistical method that was used on the other hand, for non normal distribution of datas, Wilcoxon test was used as statistical methods. ROC curve (Receiver Operating Characteristic) method was used to determine the discrimination of variables. For multivariate analysis, the factors were further analysed with the logistic regression analysis. Hosmer-Lemeshow goodness of fit statistical analysis was used to assess the model fitness. A p-value of less than 0.05 was accepted as significant.

Results

The ages of MPS patients ranged between 18-204 months (88.9 $\pm$ 48.2 months mean $\pm$ standard deviation). Ages in the control group ranged between 29-186 months (91.37 $\pm$ 42.47 mean $\pm$ standard deviation). There were no statistically significant differences in ages between MPS patients and the control group. Body mass indexes (BMI) were calculated and found that the BMI of MPS patients was 17.52 $\pm$ 4.83kg/m² and the BMI of the control group was 17.31 $\pm$ 2.86 kg/m² (Suppl File 1).

Comparison of proinflammatory cytokines in MPS and control group

All studied pro-inflammatory cytokines (IL-6, IL-1 β TNF- α) were significantly higher in patients with MPS compared to healthy individuals (p=0.000, p=0.005, p=0.000). Although it was observed that all cytokines were increased in untreated MPS patients, we only found significant differences in IL-6 levels when compared to ERT treated MPS patients (IL-6, p=0.001, IL-1 β =0.858, TNF- α p=0.067) (Table 1).

Comparison of proinflammatory cytokines in ERT treated MPS patients with pretreatment sample and control group

All cytokines were higher in patients under ERT than in the control group. It was found that IL-6 levels had no statistically significant results between patients under ERT and the control group (p=0.496). IL-1 β and TNF- α levels were significantly higher in patients under treatment compared to normal controls (IL-1 β p=0.025, TNF- α p=0.000) (Table 2).

Table1. Comparison of cytokines between MPS patients and control group [Median (IQR)]

	Patient (n=43)	Control (n=29)	P*
Age (months)	80.0 (48.0-127.0)	79.0 (57.5-132.5)	0.688
IL-6 pg/ml	30.1 (23.9-33.4)	21.1 (14.2-27.6)	0.000
IL-1 pg/ml	160.5 (151.6-177.1)	152.4 (141.1-163.4)	0.005
TNF- α pg/ml	69.7 (47.9-90.4)	26.7 (18.9-41.9)	0.000

*: Mann-Whitney U Test N: Number, p $\leq$ 0.05 is significant

Table2. Comparison of Pre-Post Treatment Values of ERT Patients and Control Group Values in MPS Patient Group [Median (IQR)]

		With ERT (n=15)	Control (n=29)	P*
Pre Treatment	IL-6 pg/ml	20.0 (14.8 - 23.5)	21.1 (14.2-27.6)	0.911
	IL-1 pg/ml	162.7 (154.9-173.4)	152.4 (141.1-163.4)	0.007
	TNF- α pg/ml	67.0 (45.3-79.3)	26.7 (18.9-41.9)	0.000
Post Treatment	IL-6 pg/ml	17.9 (13.6-27.8)	21.1 (14.2-27.6)	0.814
	IL-1 pg/ml	147.1 (128.2-157.9)	152.4 (141.1-163.4)	0.250
	TNF- α pg/ml	50.2 (40.9-56.7)	26.7 (18.9-41.9)	0.000

*: Mann-Whitney U Test

N: Number, ERT: Enzyme replacement therapy, $p \leq 0.05$ is significant,**Comparison of proinflammatory cytokines in ERT treated MPS patients with posttreatment sample and control group**

TNF- α was higher in MPS patients whose samples were taken after ERT when compared to the control group with a significant difference ($p=0.000$). The levels of IL-6 and IL-1 β were decreased after ERT and there were no significant differences between patients whose samples were obtained after ERT and normal controls (IL-6 $p=0.814$, IL-1 β $p=0.250$) (Table 2).

Comparison of proinflammatory cytokines in ERT treated and untreated MPS patients.

We investigated the proinflammatory cytokines before ERT, and after ERT. All biomarkers tended to be higher before ERT and decreased after ERT. Although IL-1 β and TNF- α levels were statistically significant between groups, there was no statistical significance in terms of IL-6 levels between ERT treated and

untreated group (for IL-1 β $p=0.005$, IL-6 $p=0.281$, TNF- α $p=0.023$) (Table 3).

Comparison of proinflammatory cytokines in pre-treatment and post-treatment MPS patients.

The proinflammatory cytokine levels were investigated, it was found that IL-1 β and TNF- α levels were significantly decreased after ERT (IL-1 β $p=0.005$, TNF- α $p=0.023$). There was no significant decrease in IL-6 after ERT ($p=0.281$) (Table 4).

ROC analysis showing the specificity and sensitivity of the cytokines

It was found that all cytokines had higher sensitivity for MPS patients (Figure 1, Supplemental File 2) although IL-6 and TNF- α had higher specificity for patients with ERT treated and untreated group (Table 5).

Table 3. Comparison of cytokines between MPS patients treated with ERT and MPS patients who did not take ERT. [Median (IQR)]

	With ERT (n=15)	Without ERT (n=28)	P*
IL-6 pg/ml	20.0 (14.8-23.5)	32.7 (29.0-33.9)	0.001
IL-1 pg/ml	162.7 (154.9-173.4)	159.8 (149.9-196.3)	0.858
TNF- α pg/ml	67.0 (45.3-79.3)	77.3 (55.3-146.4)	0.067

*: Mann-Whitney U Test

N: Number, ERT: Enzyme replacement therapy, $p \leq 0.05$ is significant**Table 4.** Intra-Post Comparison of ERT Recipients in the MPS Patient Group [Median (IQR)]

	With ERT (n=15)		P*
	Pre-Treatment	Post-Treatment	
IL-6 pg/ml	20.0 (14.8-23.5)	21.1 (14.2-27.6)	0.281
IL-1 pg/ml	162.7 (154.9-173.4)	147.1 (128.2-157.9)	0.005
TNF- α pg/ml	67.0 (45.3-79.3)	50.2 (40.9-56.7)	0.023

*: Wilcoxon Signed Ranks Test

N: Number, ERT: Enzyme replacement therapy, $p \leq 0.05$ is significant

Table 5. ROC Analysis (ERT treated and untreated)

	AUC	Cut Off	Sensitivity	Specificity	+PV	-PV	Youden Index	%95 CI	P*
IL-6 pg/ml	0.814	>26.9	89.3	80.0	89.3	80.0	0.69	0.637-0.991	0.001
IL-1 pg/ml	0.517	>177.1	32.1	93.3	90.0	42.4	0.25	0.334-0.700	0.858
TNF-α pg/ml	0.671	>70.4	60.7	80.0	85.0	52.2	0.41	0.511-0.832	0.036

*: Roc Curve Analysis

Binary Logistic regression

Logistic regression analysis was performed to investigate the effects of variables (IL-6, IL-1, and TNF) whose differences were found to be statistically significant between the MPS patients and

the control group on the presence of MPS. It has been found that IL-6 and TNF could be associated with MPS. Accordingly, it was found that those with high IL-6 could have MPS 1.16 times higher than those without, and 1.09 times higher than those without TNF (Table 6).

Table 6. Risk factors (Patient-Control))

Cytokines	OR (95% CI)	P*
IL-6 pg/ml	1.16 (1.03-1.31)	0.012
IL-1 pg/ml	1.03 (0.98-1.08)	0.295
TNF-α pg/ml	1.09 (1.04-1.14)	0.000

*: Binary Logistic Regression (Nagelkerke R² = 0.707, Hosmer and Lemeshow Test = 0.370)

Discussion

In this study, it was revealed that pro-inflammatory cytokines were important biomarkers in the follow-up of patients with MPS.

It was found that pro-inflammatory biomarkers, IL-6, IL-1 β , TNF α were significantly higher in patients with MPS compared to those in normal controls. In MPS, intralysosomal GAG deposition may lead to Toll-like receptor signaling pathway activation and cause to release of some inflammatory mediators, cytokines, growth factors, proteases, and chemokines. Some studies showed increased pro-inflammatory cytokines such as TNF- α and IL-6 production and decreased anti-inflammatory cytokines [22,23]. Wang et al reported that TNF- α production was decreased by suppressing TLR-4 signaling pathways by inhibiting the infiltration of neutrophils [24]. It has been shown that inhibition of TNF- α could reduce bone complications in models of MPS VII rats [25]. Simonaro et al reported in some animal models of MPS VI and VII suggesting that intralysosomal GAG deposition leads some cytokines, chemokines, oxidative stress mediators to release [12]. Although GAGs activate TLR-4 pathways and then cytokine expression, they can also induce cytokines independent from the TLR-4 pathway. In TLR-4 deficient mice, bone pathology was again observed [25]. In this study, pro-inflammatory cytokines including IL-1 β , IL-6, TNF- α were found higher in MPS patients compared to control groups. Increased concentration of cytokine levels suggests that GAG accumulation in tissues triggers inflammatory pathways in MPS patients as it was demonstrated before in animal models [17,26,27]. Our findings were similar to the studies that revealed the increase of TNF- α , IL-1 β , IL-6 in MPS patients.

Several reports have recently been shown that central nervous system involvement in some MPS types was also due to cytokine expression. Ausseil et al. demonstrated that heparan sulfate and ganglioside accumulation resulted in TLR-4 activation in MPS mouse models [28]. Villani et al also suggested that the inflammatory process played a major role in the brain pathology of the MPS IIIB models. Cytokines and chemokines were released and the neurodegenerative process was activated [29]. Arfi et al [26] have demonstrated that a major pathogenetic mechanism underlying neurodegeneration was inflammation in MPS III-A mice. In this study, non-treated patients were mostly MPS III and high cytokine expression in this group might be due to inflammatory pathway activation in the nervous system.

Simonaro et al showed that the management with ERT in MPS might lead to decreased TNF- α release [30]. MPS patients under ERT had higher levels of cytokines than the control group it was found that IL-6 was mild to moderately increased in patients under ERT and had a steady-state near to control group. The pre-treatment samples of ERT had higher levels of proinflammatory cytokines when compared to the post-treatment group. That result supported that ERT led to decrease proinflammatory cytokine release mostly IL-6 [31,32]. In this study, we observed significantly lower levels of TNF- α , IL-1 β in the post-treatment group. The explanation might be that the production of proinflammatory cytokines was not constant throughout the day and can be influenced by many factors such as stress that could transiently stimulate cytokine production again [33,34]. Polgreen et al showed that despite treatment with either hematopoietic stem cell therapy or ERT or both MPS I, II and VI had higher values of TNF- α compared to the control group. They suggested that higher TNF- α levels were

correlated with impaired physical examination and pain due to glycosaminoglycan-induced inflammation [35]. In this study, IL-6 levels did not decrease as much as others suggesting that major proinflammatory cytokines playing role in MPS might be TNF- α and IL-1 β . One of the main reasons to investigate the antiinflammatory role of ERT in this study was the ERT is the only management in many types of MPS. It was not found any data to distinguish any effect pre and posttreatment of ERT.

Donika et al. suggested that MPS patients had increased inflammatory and oxidative stress even under the management of ERT. They studied only one cytokine that was IL-6 for the marker of inflammation and found that IL-6 levels were higher in MPS 4-A patients than in the control group [37]. Fujitsuka et al also studied proinflammatory cytokines and found that TNF- α , IL-6 and IL-1 β were higher in patients under ERT (Fujitsuka et al). In this study TNF- α and IL-1 β were significantly decreased after ERT. It suggested that ERT firstly affects TNF- α , and then IL-1 β . Besides this, another explanation might be due to fluctuations of cytokines throughout the day or the continuation of the pro-inflammatory process. IL-6 levels might not show significant variations during the ERT period.

This study showed that in MPS patients proinflammatory cytokines might reveal the inflammation period continues during the treatment although ERT is effective management by decreasing the cytokine levels after ERT.

Currently, there is limiting clinical and observational data about the inflammatory process of MPS patients under ERT and ERT effects on altered immune function of the MPS patients. This study could support that ERT was able to reduce the cytokine levels involved in the pro-inflammatory pathway and TNF- α is the key cytokine in the pathogenesis. Beside, subtype differences of MPS patients considering enzyme replacement therapy might influence the inflammatory pathway. It might be a limitation of the study. The another limitation of the study was the small number of the patients included in the study.

Importantly clinical effects of combined treatment with ERT and anti-TNF- α therapy were significant on MPS patients. Anti TNF- α drugs could be used to decrease TNF- α levels in circulation, alleviate the inflammatory process in tissues and prevent the progression of the disease. Animals treated with anti-TNF- α drugs had normal levels of TNF- α and they had no joint and bone symptoms [30]. In a study of MPS VI models, tracheas were investigated and g-found that rats treated with ERT and anti-TNF- α drugs had significantly thinner and wider tracheas than rats that were untreated or treated with only ERT [38]. These results were attributed to the anti-inflammatory effect of anti TNF- α drugs on tissues. That anti-inflammatory mediators can regress cytokine levels in MPS patients together with ERT treatment. In this study, all cytokines revealed higher specificity for MPS patients, and TNF- α levels had higher sensitivity for the MPS group.

Conclusion

In conclusion, MPS is an inflammatory disease that GAG deposition plays a major role in the aggravation of inflammation and so on TLR-4 pathway activation. GAG deposition alone or with TLR-4 activation and other inflammatory processes causes

the activation of cascade proinflammatory mediators. ERT reduces the cytokine expression in MPS patients. It might be a possible result that this study might suggest new management strategies in MPS patients. Further investigations are needed to understand the pathologic mechanism of inflammatory process in MPS patients. The understanding of the inflammatory pathway pathogenesis in MPS may help to add antiinflammatory drugs as a treatment modality to alleviate the disease together with ERT.

Conflict of interests

The authors declare that they have no competing interests.

Financial Disclosure

All authors declare no financial support.

Ethical approval

The entire study protocol was designed according to the Helsinki Declaration and approved by the local ethical committee of Gazi University Hospital.

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