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Interleukin 1β in morphea

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Abstract

Morphea (circumscribed scleroderma, localized scleroderma) represents a disease included into the sceroderma group of autoimmune connective tissue diseases. Interleukin (IL) – 1β may play role in pathogenesis of the disease as a cytokine of pronounced pro-inflammatory properties. The study aimed at evaluation of IL-1β levels in morphea. The study was conducted on 41 blood and 29 skin samples from patients with morphea. The evaluation included: 1) expression of IL-1β gene in peripheral blood mononuclear cells (PBMC) using real-time PCR, 2) plasma levels of IL-1β using ELISA, 3) expression of IL-1β gene in skin by real-time PCR. A significantly higher expression of IL-1β in PBMC of morphea patients and higher plasma levels of the cytokine were disclosed, as compared to healthy controls. No difference was noted in expression of IL-1β in the skin between morphea patients and healthy controls.

Key words: *morphea, scleroderma, interleukin 1β, autoimmune diseases, IL-1.*

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Introduction

Morphea (circumscribed scleroderma, localized scleroderma) belongs to the group of autoimmune connective tissue diseases which includes skin thickens, and which used to be termed scleroderma. It manifests a similarity to systemic sclerosis (SSc) and a number of scleroderma-like diseases [1-5]. The most frequent varieties of morphea include morphea en plaques (MEP), generalized morphea (GM), linear morphea (LM) and deep morphea (DM) [1, 3]. The aforementioned skin induration is preceded by vascular abnormalities and inflammation [3, 6-8]. Interleukin (IL) – 1β represents a cytokine of pronounced pro-inflammatory properties, which stimulates neutrophilic and lymphocytic infiltrate at the site of tissue injury and promotes lymphocyte T-dependent antibody production [9, 10]. In parallel, the interleukin is supposed to induce proliferation of fibroblasts and to inhibit synthesis of collagen [11]. Thus, potentially it can play a role in pathogenesis of diseases progressing with fibrosis, particularly in their early, inflammatory phases. The study aimed at examination of IL-1β role in pathomechanism of morphea.

Material and methods

Patients

The study was performed on 41 patients with morphea [mean age of 44 years; standard deviation (SD) = 18; 22 women, 19 men], for whom the diagnosis was confirmed by histological examination. In 20 patients MEP, in 14 GM and in 7 LM was diagnosed. Blood was sampled from every person of the studied groups. Skin samples were obtained from morphea lesions of 29 patients (mean age of 42 years; SD = 18; 12 women, 17 men). None of the patients had received topical or systemic treatment except topically applied emollients. Control group included 47 healthy volunteers (mean age of 33 years, SD = 9; 35 women, 12 men)

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from whom blood was sampled and 13 women patients with breast carcinoma, which manifested no skin involvement (mean age of 52 years, SD = 18 years), from whom healthy skin samples were taken following mastectomy procedure. The study protocol received consent of the local Bioethical Committe.

Around 5 ml of blood was sampled to EDTA-containing vacutainers (*Monovette, Sarstedt*). Peripheral blood mononuclear cells (PBMC) and plasma were obtained by centrifugation of the blood over Ficoll density gradient (*Ficoll-Histopaque* 1.070 g/cm^3, *Sigma Diagnostics, Inc. St. Louis, USA*). Until examined, the plasma was kept at the temperature of –80°C.

Directly after isolation, skin samples were frozen in liquid nitrogen and stored at the temperature of –80°C.

Evaluation of activity of the disease

Patients, in whom within the recent 6 months appearance of new lesions, spread of previously existing ones or presence of erythematous margins were noted, were qualified to the group with an active process [12, 13].

Enzyme linked immunosorbent assay (ELISA)

Examination of plasma IL-1β concentration by ELISA was conducted using commercially available ELISA kits (R&D System, Minneapolis, USA). The estimations were conducted as recommended by the manufacturer.

Expression of IL-1β genes in PBMC

Total RNA, isolated from PBMC, was obtained with the use of a modified Chomczyński and Sacchi protocol [14]. In order to avoid contamination with genomic DNA, a commercially available kit containing recombinant DNase I (Ambion, USA) was used. In every case 1 μg RNA was taken for the reaction. Reverse transcription (RT) was performed with a commercially available kit (Roche Applied Science). cDNA samples obtained by RT were subjected to quantitative analysis of expression using real-time PCR. The analyses took advantage of the Light Cycler 2 system (Roche Diagnostics GmbH, Germany) and commercially available kit for real-time PCR, containing Sybr Green (SG) dye (Roche Applied Science).

The analysis involved a selected IL-1β mRNA fragment of 157 base pairs (bp) in length, corresponding to chromosome 2q14. The amplicon spreads from exon 5 to 7, which with an appropriately short elongation time limited the chance for amplification of undesired sequences of genomic origin. Efficacy of DNase I action and, thus, efficacy of elimination of genomic DNase I contamination, was confirmed by real-time PCR on the material not subjected to reverse transcription (negative no-RT control). Primers for IL-1β were obtained from the on-line database of the primers http://www.rtprimerdb.org/ [15-17] (Table 1).

Real-time PCR was optimized, adjusting amplification parameters and the composition of reaction to obtain possibly best results. Size of the product was evaluated by electrophoresis in an agarose gel with SYBR Safe stain (Invitrogen). The reaction was regarded optimized when a single band of correct size was present in an agarose gel and when the analisis of amplicon melting temperature following real-time PCR pointed to a single and a homogenous amplification product. The quantitation of studied transcript was conducted using standard curve, prepared for samples of synthetic DNA of known concentration. The obtained results were expressed in copy numbers per million copies of the reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference gene. Efficacy of using this gene as the reference in this type of studies has been justified in literature [18].

Expression of IL-1β gene in skin

Isolation of total RNA from skin biopsies was washes in isopropanol and 75% ethanol. Additionally, all preparations were subjected to digestion with a recombinant DNase I. All the stages of expression evaluation were identical to those used in the case of PBMC material.

Evaluation of anti-nuclear antibodies

Anti-nuclear antibodies (ANA) titres were established by indirect immunofluorescence on a mosaic of substrates (neoplastic cell line of laryngeal carcinoma, HEp-2 / monkey hepatocytes) employing a commercially available kits (Euroimmun, Germany).

Statistical analysis

Statistical analysis was performed with the use of Statistica 9.1 software (Statsoft). In order to describe variability within the studied group, its subgroups and control groups appropriate descriptive statistics were prepared, including mean and SD for age and duration of the disease

Table 1. Primers used in the study

Name	5'-3' sequence	Length of amplicon [bp]	Source of base sequence
GAPDH-F	CTGCACCACCAACTGCTTAG	105	Ensembl: ENST00000229239 Glyceraldehyde-3-phosphate dehydrogenase [16]
GAPDH-R	TTCTGGGTGGCAGTGATG		
IL1B-F	TGGAGCAACAAGTGGTGT	157	http://www.rtprimerdb.org/ assay id=3054 [17]
IL1B-R	TTGGGATCTACACTCTCCAGC		

and, in view of the unfulfilled assumptions as to the normal distribution of the data, median, minimum and maximum values for the remaining analysed variables. Significance of differences between the studied group and respective controls was evaluated using the Mann-Whitney U test. Evaluation of significance of differences in variables distribution in individual subtypes of the disease took advantage of the ANOVA Kruskal-Wallis test. For description of relationships between duration of the disease and the analysed variables, both in subgroups and in the entire group, Spearman's rank correlation method was used. The results were regar ded significant at $p < 0.05$. Graphically, the results were presented in box graphs.

Results

An active morbid process was noted in 26 patients (10 with MEP, 12 with GM, 4 with LM), an inactive form of the disease was noted in 15 persons (10 with MEP, 2 with GM and 3 with LM). In the entire studied group mean duration of the disease amounted to 5.4 years (SD = 8.0), in MEP 3.4 years (SD = 4.5), in GM 3.9 (SD = 3.0), in LM 14.7 (SD = 17.3) (Table 2).

Plasma concentration of IL-1β, its expression in PBMC and in skin of the studied and control individuals are presented in Table 3. The minimum detectable concentration was 0.05 pg/ml. IL-1β was found more frequently in plasma of morphea patients than in healthy control group. Plas-

Table 2. Values of descriptive statistics for activity of the disease course and duration of the disease in the studied group

		Morphea	Morphea subtypes		
			MEP	GM	LM
number of patients	*n*	**41**	20	14	7
duration of the disease	mean (years)	**5.4**	3.4	3.9	14.3
	SD	**8.0**	4.5	3.0	17.3
active / inactive disease		**26/15**	10/10	12/2	4/3

MEP – morphea en plaque, GM – generalised morphea, LM – linear morphea, n – number of patients, SD – standard deviation, min – minimum value, max – maximum value

Table 3. Expression of IL-1β in PBMC, its plasma concentration and expression in skin in the experimental group, control group and in examined subgroups

		Morphea	MEP	GM	LM	Control
expression in PBMC (per million copies of GAPDH)	median	9345	9912	8011	8294	3442
	min	2445	2695	3351	2445	233
	max	36230	33088	20892	36230	17348
	n	41	20	14	7	47
	p	$p < 0.001$*	$p < 0.001$*	$p = 0.001$*	$p = 0.030$*	–
plasma concentration (pg/ml)	median	0.0	0.1	0.0	0.0	0.0
	min	0.0	0.0	0.0	0.0	0.0
	max	2	2	1	0.3	1.6
	n	41	20	14	7	47
	p	$p = 0.002$*	$p < 0.001$*	$p = 0.183$	$p = 0.645$	–
expression in skin (per million copies of GAPDH)	median	686	728	367	438	2146
	min	0.0	0.0	0.0	302	0.0
	max	24775	5050	24775	1927	18866
	n	29	13	10	6	13
	p	$p = 0.183$	$p = 0.248$	$p = 0.250$	$p = 0.273$	–

*PBMC – peripheral blood mononuclear cells, MEP – morphea en plaque, GM – generalised morphea, LM – linear morphea, n – number of patients, p – value of p as related to the control group, * significant difference*

Table 4. ANA evaluated by indirect immunofluorescence on HEp-2 cells

ANA titre	Morphea (n = 41)		MEP (n = 20)		GM (n = 14)		LM (n = 7)	
	n	%	n	%	n	%	n	%
0	8	19.5	4	20.0	3	21.4	1	14.3
1/80	13	31.7	6	30.0	6	42.9	1	14.3
1/160	7	17.1	4	20.0	2	14.3	1	14.3
1/320	6	14.6	5	25.0	0	0	1	14.3
1/640	3	7.3	0	0	2	14.3	1	14.3
1/1280	3	7.3	0	0	1	7.1	2	28.6
1/2560	1	2.4	1	5.0	0	0	0	0

ANA – anti-nuclear antibodies, MEP – morphea en plaque, GM – generalised morphea, LM – linear morphea, n – number of patients

ma value higher or equal 0.05 pg/ml was detected in 14 of 41 morphea samples (34.2% of cases), 10 of 20 MEP samples (50%), 3 of 14 GM samples (21.4%), 1 of 7 LM samples (14.3%) and 4 of 47 control samples (8.5%).

Results of the indirect immunofluorescent testing presence and titre of ANA are shown in Table 4.

A significantly increased expression levels of IL-1β were distribution noted in PBMC of morphea patients in the entire examined group ($p < 0.001$) (Fig. 1) and in all the subgroups: MEP ($p < 0.001$), GM ($p = 0.001$), LM ($p = 0.03$) as compared to healthy control. A significantly increased plasma concentration of IL-1β was noted in the entire group of morphea patients ($p = 0.002$) (Fig. 2) and in MEP patients ($p < 0.001$), but not in GM or LM patients, as compared to healthy controls. No significant difference was noted in cutaneous expression of IL-1β between morphea patients (nor its subgroups) and the control group (Table 3).

Analysis of the entire examined group did not disclose any correlation between expression of IL-1β in PMBC, its plasma concentration or expression in skin on one hand and studied variables, including duration of the disease, its activity and ANA titre on the other. In the subgroup of patients with LM a direct relationship was disclosed between expression of IL-1β in PBMC and titre of ANA ($\rho = 0.75$ at $p = 0.049$) and between expression of IL-1β in skin and activity of the disease ($\rho = 0.87$ at $p = 0.02$). In the subgroup of GM patients an inverse relationship was documented between plasma concentration of IL-1β and duration of the disease ($\rho = -0.59$ at $p = 0.02$).

Discussion

IL-1β, belonging to the IL-1 family, represents a cytokine widespread in the body, with strong pro-inflammatory properties. Monocytes and macrophages are its prin-

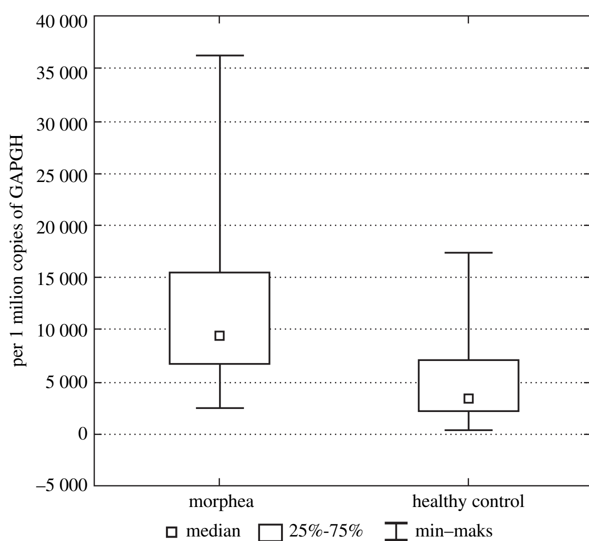

Fig. 1. Expression of IL-1β in PBMC of morphea patients

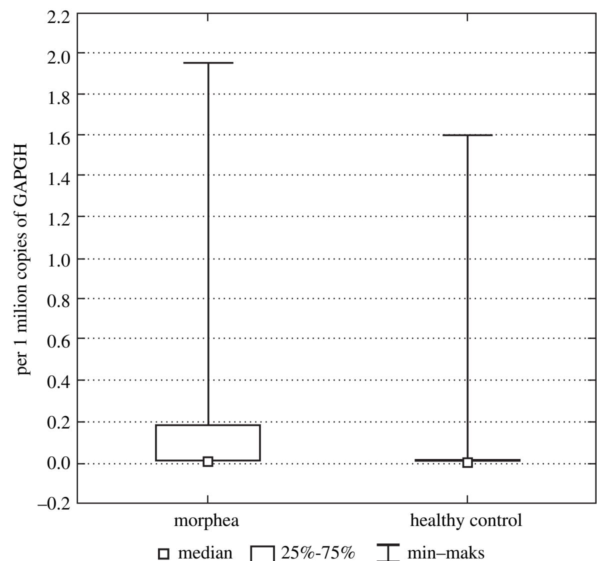

Fig. 2. Plasma concentration of IL-1β in morphea patients

cipal source [9, 10]. Those cells have been noted to be the first cellular components of inflammatory infiltrate in morphea [8, 19, 20]. The final activation of IL-1β is thought to depend on post-translational enzymatic processing of pro-IL-1β, executed by caspase 1, which, in turn, depends on the activation of inflammosomes [9, 10]. Interestingly, activation of the NLRP3 inflammosome by the particles of asbestos and silica is supposed to play a role in pathogenesis of pulmonary fibrosis [21]. Activation of inflammosomes and, thus, release from the cells of vast amounts of IL-1β is supposed to be the key phenomenon in the course of autoinflammatory diseases, such as e.g., familial Mediterranean fever [22]. Nevertheless, morphea does not fulfil the classic diagnostic criteria of this group of diseases, due to e.g., the high incidence of ANA manifestation [22, 23], which was also demonstrated by the results of this study. The significance of IL-1β is also accentuated in several autoimmune diseases, such as adult onset Still disease, systemic juvenile idiopathic arthritis, rheumatoid arthritis or Crohn's disease [22].

In this study we examined the expression of the IL-1β gene in PBMC and skin, as well as IL-1β plasma concentration in morphea patients. It should be stressed that untill now the only serum level of IL-1β was evaluated in morphea patients. Results of the studies demonstrated a significantly higher expression of IL-1β gene in PBMC of morphea patients, as compared to the healthy control group, irrespectively whether they suffered from MEP, GM, or LM. In addition a significantly increased plasma concentration of IL-1β was documented in the entire examined group and in the MEP subgroup, as compared to the healthy control group. In the GM and LM subgroups the plasma concentration of the cytokine was also higher but the difference failed to reach statistical significance. However, it should be added that the subgroups manifested lower numerical force than the MEP subgroup, which might have affected the results. A significantly higher IL-1 concentration in serum of morphea patients was observed also by Alecu *et al.* [24]. On the other hand, the results presented by us demonstrated no significant difference in the level of IL-1β expression in skin of morphea patients, as compared to the control group. Nonetheless, the expression in skin of patients with LM showed a correlation with activity of the morbid process. Since the active phase of morphea is linked to the presence of inflammatory infiltrate [3, 6, 8], this may suggest involvement of the cytokine just in the early, inflammatory stages of the disease. The observation has been additionally confirmed by the demonstrated inverse correlation between plasma level of IL-1β in the group of patients with GM and duration of the disease, which means that higher concentrations of the cytokine are encountered just in the early / inflammatory phases of the disease. In addition, a direct relationship has been demonstrated between the expression of IL-1β in the LM subgroup and ANA titre, even if it should be kept in mind that the subgroup was small, which might have affected the results.

In the context of the obtained results, it is worth noting that clinical trials are currently underway with IL-1β blocking drugs in treatment of some autoimmune diseases, such as the aforementioned adult onset Still disease, systemic juvenile idiopathic arthritis, rheumatoid arthritis, with promising results [22]. The group of biological drugs capable of blocking IL-1β include anakinra (recombined human IL-1 receptor), rilonacept (dimeric protein consisting of the extracellular fragment of IL-1 receptor and Fc fragment of IgG1), or kanakinumab (a monoclonal antibody specific for IL-1β) [22]. On the other hand the current methods of managing morphea, particularly of its more severe forms, are limited and frequently futile. Moreover, not many placebo-controlled multicentre trials are conducted, which could confirm efficacy of such approaches. Currently, the most accepted method is to use phototherapy, methotrexate and glucocorticoids [3, 25-27]. Obviously, one has to keep in mind the potentially self-restricting course of the disease and the fact that in most cases of MEP it does not seem reasonable to use treatments other than emollients. The treatment should not pose higher risk for a patient than the disease itself [3, 25-27].

Summing up the above, it should be concluded that 1) IL-1β plays a role in morphea, 2) one can speculate that it plays its role particularly in the active phase of disease as the authors observed higher IL-1β plasma level in the earlier phases of GM, 3) in the context of recent achievements in pharmacotherapy, the obtained results could provide a stimulus for further considerations of the novel therapeutic potential of IL-1β blocking drugs in morphea. Nevertheless, we cannot dismiss certain aspects which decide about the restricted character of our study. Apart from the aforementioned small numerical force of studied groups it should be noted that perhaps more than one reference gene should be analyzed in the case of this study in order to improve the reliability of the results. In addition it would be advantageous to pay attention to two further possible directions of studies, stemming from the results we have presented. Firstly, it would be interesting to examine inflammosomes in the studied group of patients. Secondly, taking into account results presented by Luzin *et al.*, who detected an augmented expression the IL-1 receptor antagonist (IL-1RA), but not of IL-1β in cells of bronchopulmonary lavage in patients with systemic scleroderma [28], perhaps it would be worth examining the expression of the protein also in morphea patients.

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