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The Effect of Methotrexate and Leflunomide on the Cytokine Profile and Nitric Oxide Metabolism in Rheumatoid Arthritis Patients

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Abstract

Objective: To reveal the role of individual cytokines and nitric oxide in pharmacological effects of methotrexate and leflunomide in rheumatoid arthritis (RA) patients.

Materials and methods: Ninety four patients with confirmed seropositive RA aged 20-60 yrs were examined. The patients were separated into groups of early and late RA and of "erosive" and "non-erosive" RA. 63 patients received methotrexate, and 31, leflunomide. The levels of IL-1β, IL-6, IL-10, and TNF-α were determined in blood serum and synovial fluid using solid-phase enzyme immunoassay. The levels of NO2 and NO3 were determined in blood serum, synovial fluid, and urine by spectrophotometry. All analyses were performed both before and 6 months post therapy. Therapy effectiveness was estimated in the same period.

Results: The levels of NO metabolites and cytokines except for IL-6 were increased in synovial fluid and urine of all patients compared to the control. The methotrexate therapy resulted in an increase in IL-1, TNF, and NO metabolites in the peripheral blood. As distinct from methotrexate, leflunomide strongly suppressed IL-6 production in blood serum. The therapy using either drug resulted in a manifold decrease in the levels of IL-1, TNF, and NO metabolites in synovial fluid.

Conclusions: There were opposite changes of levels of cytokines and NO metabolites in RA patient's blood and synovial fluid receiving methotrexate and leflunomide respectively.

Keywords: Rheumatoid arthritis; Nitric oxide; Cytokines; Methotrexate; Leflunomide

Introduction

Pathogenetic therapy of rheumatoid arthritis (RA) is a complex task. According to modern views on the development of autoimmune inflammation in RA, cytokine disbalance plays a great part in RA pathogenesis [1-3]. The role of NO in joint pathology is supported by several experimental and clinical laboratory studies; however, its exact role in RA remains obscure [4-6]. The mechanisms of pharmacodynamic effects of of disease-modifying antirheumatic drugs (DMARDs) are only partly known. The problems associated with RA pharmacotherapy include primary resistance, poor effectiveness of formerly effective drugs, and side effects [7]. In most cases, as few as one third of RA patients can receive therapy with the same DMARD longer than four years [8]. Thus, new approaches to pharmacotherapy should be developed, the mechanisms by which known drugs act should be further studied, and reliable indicators of therapy effectiveness should be found.

Purpose

To find out the role of cytokines and NO in methotrexate and leflunomide pharmacodynamics in RA.

Materials and Methods

A prospective cohort study of 94 seropositive RA patients was performed. In all patients, the diagnosis complied with ARC/EULAR diagnostic criteria for RA (2010). The sample comprised patients with active RA, with indication for DMARD therapy, without contraindication for methotrexate and leflunomide, and without cognitive disorders. The patients did not receive DMARD therapy for two months and received NSAID therapy for two weeks prior to study entry. The sample comprised 87 women and 7 men 20-60 yrs old. Two groups of patients were distinguished, those with early and late RA (RA duration less or more than 3 months, respectively) [9]. Erosive RA was diagnosed by the presence of erosion zones in bone structures formed within 24 months after disease onset, and non-erosive RA, by the absence of erosion [10,11]. The RA activity was middle and high in 46.7 and 52.3% of the patients, respectively. The distribution of the patients by roentgenological stages was as follows: stage I - 2.3%, II - 27.35%, III - 46.25%, and IV - 24.10% according to Steinbroker [12]. Most patients had grade 2 and 3 functional joint impairment (ARA, 1992). The groups did not differ significantly in clinical or demographic parameters.

Depending on clinical and laboratory RA activity 63 patients received methotrexate (20 mg, once a week, intramuscular injection) and 31, leflunomide (oral, 100 mg/day for the first three days, then 20 mg/day). DMARD therapy effectiveness was estimated six months after therapy onset using Disease Activity Score 28 (DAS 28) [13]. A signed Information Consent Form was obtained from each patient. The study was approved by the Independent Interdisciplinary Ethics Committee of the Vladivostok State Medical University.

Synovial fluid samples were obtained in the course of arthroscopy and synovectomy performed in accordance with indications (continuously recurrent knee-joint synovitis for at least 2 months). The control samples of synovial fluid were obtained from 20 patients (eight men and twelve women aged 25-55 years) at exploratory arthrotomy of their knee joints indicated by alleged meniscus injury. Samples of

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blood serum and synovial fluid were frozen at -70°C immediately after sampling.

The levels of IL-1β, IL-6, IL-10, and TNF-α were determined in blood serum and synovial fluid by solid-phase enzyme immunoassay using BioSource International Kits. The levels of nitrite and nitrate were determined by spectrophotometry in blood serum, synovial fluid, and urine. All analyses were repeated six months post therapy.

All statistical tests were performed using the Statistica 6.0 (Statsoft) package.

Results

Solid-phase enzyme immunoassay revealed a substantial increase in the levels of cytokines in synovial fluid of RA patients compared to the control (Table 1).

The levels of cytokines except for IL-6 in blood serum of RA patients did not exceed those in the control. Noteworthy, the levels of IL-6 in system blood flow and synovial fluid were considerably higher in the RA patients compared to the control, mainly, due to late RA patients. This suggests the role of IL-6 in chronization of inflammation at RA [14-16].

The level of IL-1 in synovial fluid was only increased in early RA patients compared to the control (Table 1). The levels of TNF and IL-10 in synovial fluid were significantly greater in early RA patients further increasing in late RA (Table 1).

The level of IL-10 was significantly negatively correlated with that of rheumatoid factor (RF) ($r=-0.67$; $p<0.05$). The level of IL-10 in synovial fluid of "erosive" RA patients was increased and was correlated with roentgenological stage of RA ($r=0.58$; $p<0.05$).

The levels of NO metabolites in synovial fluid and urine, but not in blood serum, of RA patients were significantly greater compared to the control (Table 2).

The local levels of nitrites in "erosive" RA patients were increased compared to the control suggesting an active role of NO in aggravating tissue destruction at RA (Table 2).

Methotrexate therapy proved to be more effective especially in early RA patients and correlate very accurately with the DAS 28 lowering. It had diverse effects on the cytokine profile. The levels of IL-1 and TNF were increased in blood serum and decreased considerably in synovial fluid (Table 3). Interestingly, the level of TNF in blood serum was only increased in "erosive" RA patients. The levels of NO metabolites were increased in the peripheral blood flow and decreased considerably in synovial fluid (Table 4). The local levels of nitrates were most prominently decreased in early RA patients.

Leflunomide therapy resulted in a considerable reduction in DAS 28. We suggest that anti-inflammatory effects of leflunomide occur via suppression of the production of IL-1 and TNF and stimulation of the production of IL-10 in synovial membrane (Table 3). As distinct from methotrexate therapy, the level of IL-6 in blood serum was decreased. The level of IL-10 was also considerably decreased (Table 3). The levels of NO metabolites were decreased both in blood serum and synovial fluid (Table 4).

Discussion

It seems that the levels of NO metabolites and cytokines except for IL-6 in RA patients differed from the control only in synovial fluid. We showed that methotrexate and leflunomide differ in their targets and effects at both local and global level.

Although an increase in the level of TNF in the system blood flow was not correlated with anti-inflammatory effectiveness of methotrexate, increase in the levels of IL-1 and TNF-α in blood serum may underlie secondary resistance to methotrexate. It seems that elevation of IL-1 and TNF-α in blood sample after 6 months of methotrexate treatment is a certain type of biologic adaptation. It is desirable to trace the level of IL-1, TNF-α against the background of methotrexate therapy monthly. It is not improbable that short period of cytokines depletion is followed by cytokines elevation. With respect

Item (pg/ml)	Control n=20	All RA patients n=94	Early RA patients n=22	Late RA patients n=41
IL-1-ser	1,65±0,03	0,86±0,17	0,25 ± 0,30*	0,96 ± 0,20*◊
IL-6-ser	2,47±0,03	60,22±3,40 *	7,32 ± 4,33	104,55 ±41,20*◊
IL-10-ser	0,21±0,02	0,50±0,23	0,25 ± 0,23	0,45 ± 0,39
TNF-ser	2,17±0,07	3,69±1,18	3,97 ± 1,93	3,06 ± 1,46
IL-1-sin	1,95±0,91	10,06±2,28*	13,87 ± 2,60*	0,60 ± 0,40 *◊
IL-6-sin	3,48±1,12	1431,77±110,96*	1505,00±182,04*	1745,00±35,53*
IL-10-sin	1,11±1,02	7,26±1,18*	4,30 ± 2,60	10,82 ± 5,17*◊
TNF-sin	4,12±2,03	150,59±15,61*	101,00 ± 80,00*	158,21 ± 8,05*

Note: n — number of patients in each group. * — differences from the control were significant at $p<0.05$

Table 1: Cytokine profile in rheumatoid arthritis patients (mean ± SEM).

Item (μM/ml)	Control n=20	All RA patients n=94	Non-erosive RA patients n=18	Erosive RA patients n=45
NO2-ser	1,30±0,11	2,46±0,33	1,76 ± 0,51	2,25 ± 0,42
NO3-ser	1,17±0,08	1,13±0,24	0,86 ± 0,43	0,99 ± 0,22
NO-ser	2,47±0,10	3,54±0,48*	2,62 ± 0,79	3,24 ± 0,55
NO2-m	1,12±0,31	9,19±1,25	6,42 ± 1,20*	9,95 ± 2,33*
NO3-m	0,58±0,09	15,06±3,01	17,05 ± 4,16*	17,97 ± 4,42*
NO-m	1,71±0,12	24,25±3,07*	23,47 ± 4,76*	27,92 ± 3,81*
NO2-sin	1,08±0,07	9,35±1,34*	7,00 ± 0,93*	8,20 ± 2,64*
NO3-sin	0,58±0,04	8,64±3,56	6,80 ± 1,20	18,03 ± 4,54*
NO-sin	1,66±0,11	17,99±3,41*	13,80 ± 1,21*	26,23 ± 3,78*◊

Note: n — number of patients in each group. * — differences from the control were significant at $p<0.05$

Table 2: Nitric oxide metabolites in rheumatoid arthritis patients (mean ± SEM).

Item **(pg/ml)**	Control **n = 20**	Methotrexate n = 63	Leflunomide n = 31
IL-1-ser	1,65 ± 0,03	BT 0,69 ±0,16* AT 1,77 ± 0,73	BT 1,40 ± 0,14 AT 1,03 ± 0,13
IL-6-ser	2,47 ± 0,03	BT 80,59 ± 35,17 AT 53,96 ± 27,17	BT 56,60 ± 12,54* AT 31,11 ± 3,66*
IL-10-ser	0,21 ± 0,02	BT 0,25 ± 0,17 AT 0,31 ± 0,24	BT 4,81 ± 0,13* AT 1,10 ± 0,02*◊
TNF-ser	2,17 ± 0,07	BT 3,40 ± 1,47 AT 31,94 ± 8,37*◊	BT 5,34 ± 1,12 AT 6,42 ± 1,54
IL-1-sin	1,95 ± 0,91	BT 11,12 ± 3,36 AT 0,65 ± 0,12 ◊	BT 12,55 ± 2,87* AT 4,77 ± 1,38 ◊
IL-6-sin	3,48 ± 1,12	BT 1446,76±124,84* AT 1820,34 ±23,65*	BT 1670,54 ± 89,44* AT 1856,49 ± 76,88*
IL-10-sin	1,11 ± 1,02	BT 7,68 ± 1,43 AT 43,80 ± 2,62*◊	BT 14,87 ± 1,55* AT 64,33 ± 1,81*◊
TNF-sin	4,12 ± 2,03	BT 164,51 ± 18,45* AT 25,50 ± 3,76*◊	BT 170,45 ± 12,77* AT 22,87 ± 2,02*◊

Note: n — number of patients in each group. * — differences from the control were significant at p<0.05. ◊ — differences between the groups were significant at p<0.05. BT – before treatment. AT – after treatment

Table 3: Cytokine profile in rheumatoid arthritis patients before and after methotrexate and leflunomide therapy (mean ± SEM).

Item (μM/ml)	Control n=20	Methotrexate n=63	Leflunomide n=31
NO2-ser	1,30 ± 0,11	BT 2,44 ± 0,68 AT 3,33 ± 0,59	BT 1,38 ± 0,31 AT 0,25 ± 0,13* ◊
NO3-ser	1,17 ± 0,08	BT 1,13 ± 0,28 AT 2,01 ± 0,58	BT 0,61 ± 0,27 AT 0,42 ± 0,11*
NO-ser	2,47 ± 0,10	BT 3,49 ± 0,54 AT 5,35 ± 0,95	BT 2,03 ± 0,18 AT 0,67 ± 0,12* ◊
NO2-m	1,12 ± 0,31	BT 9,31 ± 1,66* AT 14,21 ± 1,07*	BT 10,06 ± 2,79* AT 9,54 ± 1,32*
NO3-m	0,58 ± 0,09	BT 15,55 ± 3,75* AT 20,34 ± 2,23*	BT 12,35 ± 8,15* AT 10,54 ± 3,54*
NO-m	1,71 ± 0,12	BT 24,86 ± 4,01* AT 34,45 ± 1,75*	BT 22,42 ± 6,18* AT 20,08 ± 2,76*
NO2-sin	1,08 ± 0,07	BT 7,94 ± 1,46* AT 2,56 ± 0,54 ◊	BT 6,34 ± 1,22* AT 2,32 ± 1,43 ◊
NO3-sin	0,58 ± 0,04	BT 12,22 ± 6,21* AT 0,23 ± 0,02	BT 14,32 ± 3,34* AT 5,77 ± 1,22*
NO-sin	1,66 ± 0,11	BT 0,16 ± 4,47* AT 2,79 ± 1,23 ◊	BT 20,66 ± 4,87* AT 8,09 ± 1,55*

Note: n — number of patients in each group. * — differences from the control were significant at p<0.05. ◊ — differences between the groups were significant at p<0.05. BT – before treatment. AT – after treatment

Table 4: Nitric oxide metabolites rheumatoid arthritis patients before and after methotrexate and leflunomide therapy (mean ± SEM).

to the level of synovial fluid cytokines it could be qualified as a tissue result of biologic therapy that can not be subject to rapid fluctuations. An increase in the level of IL-10 in synovial fluid after methotrexate therapy seems to eliminate disbalance between Th1 and Th2 cytokines [17].

Of a special interest is dynamics of NO metabolites in urine under methotrexate therapy (Table 4). Anti-inflammatory effect of methotrexate is mediated by adenosine, a strong anti-inflammatory agent whose action is in turn mediated by a special system of membrane receptors [18]. Recent clinical studies showed that adenosine induces NO production in endothelium cells [19]. It was also shown that adenosine-induced vasodilatation is accompanied by an increase of NO production in endothelium cells [20]. The authors claim in the conclusion that stimulation of purinergic receptors in endothelium cells can directly induce NO production in arteries resulting in cardioprotective effects. Given this, reduction in mortality due to cardiovascular disease in RA patients attributed to methotrexate therapy [21,22] may be accounted for not only by anti-inflammatory effects of methotrexate but also, and first of all, by methotrexate-induced increase in NO level in the peripheral blood flow regardless hypercysteinemia. In this context increasing excretion of NO metabolites with urine under methotrexate treatment in patients with RA is not surprisingly and adequately depicts mechanism of methotrexate action. In contrast, decreasing level of NO metabolites in synovial fluid after methotrexate treatment reflects the final tissue result of treatment with methotrexate reflecting reduction of synovial membrane inflammation.

Interestingly, leflunomide, but not methotrexate therapy resulted in a decrease in the IL-6 level in the system blood flow. This suggests that these drugs differ in their effects on the cytokine profile bearing in mind the role of IL-6 in inflammation chronization at RA. Leflunomide offers an alternative with comparable efficacy to methotrexate as both monotherapy and, as preliminary data suggest, in combination with certain biologics agents [23]. The effects of various disease-modifying anti-rheumatic drugs could be related to it specifics of influence on innate and adaptive immunity. For instance, methotrexate and leflunomide has a different effect on CD4(+)CD25(+) regulatory T cells function. Leflunomide inhibited the anti-proliferative function of CD4(+)CD25(+) regulatory T cells on cocultured CD4(+)CD25(-) effector T cells and reduced Treg expression of Foxp3 mRNA, whereas methotrexate did not [24]. Studying particular effects of different DMARDs may suggest their effective therapeutical combinations.

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