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iNOS Expression in Peripheral Blood Leukocytes of Patients With Early Arthritis and Established Rheumatoid Arthritis

Aktywność iNOS w leukocytach krwi obwodowej u chorych na reumatoidalne zapalenie stawów i wczesne zapalenie stawów

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Abstract

Background. Nitric oxide (NO) is a small, very reactive molecule involved in many physiological processes; including immune response. The present authors previously reported increased levels of NO metabolites in the serum of patients with rheumatoid arthritis (RA) and osteoarthritis.
Objectives. The aim was to evaluate the activity of iNOS in peripheral blood leukocytes (PBLs) in patients with early arthritis (EA) and established RA and to compare it with parameters of disease activity used in clinical practice.
Material and Methods. Thirty-five patients who met the ACR criteria for RA, 31 patients with EA, and 30 healthy controls were enrolled in the study. The activity of iNOS was measured by an immunocytochemical method in PBLs separated by density gradient.
Results. The mean age of RA patients was 55.7 years. High disease activity (defined as DAS28 > 5.1) was detected in 18 patients and mean DAS28 was 5.0. Mean disease duration was 13.0 years. The mean age of EA patients was 44.1 years. High disease activity was detected in 18 patients and mean DAS28 was 5.2. Mean disease duration was 6.5 months. There was a statistically significant ($p < 0.05$) difference between RA and EA patients and healthy subjects in iNOS expression (25.4 ± 15.2%, 18.7 ± 12.3%, and 13.8 ± 15.1%, respectively). In EA the mean iNOS expression was higher in patients with cigarette abuse than in nonsmokers (22.9% vs. 14.4%), whereas no such a difference was observed in established RA. In established RA, iNOS expression correlated with CRP level, the number of tender and swollen joints, and DAS28. In EA, no correlation between iNOS expression and the analyzed parameters of disease activity was found.
Conclusion. As the difference between iNOS expression in PBLs in patients with RA and with EA cannot be attributed to differences in disease activity (DAS28 score, ESR, and CRP level are similar in both groups), further observation is needed to determine whether higher iNOS expression in some patients with early arthritis can predict future RA development. The higher iNOS expression in smokers with EA needs further study aimed at determining if smoking induces iNOS activity and thereby promotes RA development (**Adv Clin Exp. Med 2008, 17, 4, 415–421**).

Key words: inducible nitric oxide synthase (iNOS), rheumatoid arthritis (RA), early arthritis (EA).

Streszczenie

Wprowadzenie. Tlenek azotu (NO) jest małą, bardzo aktywną chemicznie cząsteczką, która jest zaangażowana w wiele procesów fizjologicznych, m.in. odgrywa istotną rolę w regulacji procesów immunologicznych. Autorzy uprzednio opisywali podwyższone stężenie metabolitów NO w surowicy chorych na reumatoidalne zapalenie stawów i chorobę zwyrodnieniową stawów.
Cel pracy. Ocena aktywności iNOS w leukocytach krwi obwodowej pobranej od chorych na r.z.s. i w.z.s.
Materiały i metody. Do badania włączono 35 chorych z ustalonym rozpoznaniem r.z.s., 31 chorych na w.z.s. i 30 zdrowych ochotników, jako grupę kontrolną. Ekspresję iNOS w leukocytach określano metodą immunocytochemiczną.

Wyniki. Średnia wieku chorych na r.z.s. wynosiła 57,7 lat, a chorych na w.z.s. 44,1 lat. Wysoką aktywność choroby definiowaną jako DAS28 > 5,1 stwierdzono u 18 chorych na r.z.s. (średnia wartość DAS28 w tej grupie wynosiła 5,0) i u 18 chorych na w.z.s. (średnia wartość DAS28 w tej grupie wynosiła 5,2). Średni czas trwania choroby wynosił w grupie chorych na r.z.s. 13,0 lat, a w grupie chorych na w.z.s. 6,5 miesiąca. Ekspresja iNOS w grupie chorych na r.z.s., w.z.s. i w grupie kontrolnej wynosiła odpowiednio 25,4% ± 15,2%, 18,7% ± 12,3% i 13,8% ± 15,1%, Wykazano istotną statystycznie różnicę między poszczególnymi grupami ($p < 0,05$). Wśród chorych na w.z.s. aktywność iNOS była istotnie wyższa u chorych palących papierosy (22,9% *vs.* 14,4%). Nie obserwowano analogicznej zależności w grupie chorych na r.z.s. W grupie chorych na r.z.s. stwierdzono istotną statystycznie korelację między ekspresją iNOS a stężeniem CRP, liczbą bolesnych i obrzękniętych stawów oraz DAS28. W grupie chorych na w.z.s. nie stwierdzono korelacji między ekspresją iNOS a wskaźnikami aktywności choroby.
Wnioski. Różnica aktywności iNOS w grupach chorych na r.z.s. i w.z.s. nie wynika z różnic w aktywności choroby, gdyż DAS28, OB i CRP są zbliżone w badanych grupach. Potrzebne są dalsze badania, które pozwolą na wyjaśnienie mechanizmów odpowiedzialnych za obserwowaną różnicę. Zwiększona aktywność iNOS u palaczy z w.z.s. wymaga także dalszych badań (**Adv Clin Exp. Med 2008, 17, 4, 415–421**).

Słowa kluczowe: indukowana syntaza tlenku azotu (iNOS), reumatoidalne zapalenie stawów (r.z.s.), wczesne zapalenie stawów (w.z.s.).

Rheumatoid arthritis (RA) is a severe progressive autoimmune condition that can lead to disability, impaired quality of life and, in some cases, reduced life expectancy. The etiopathogenesis of the disease is still unknown; therefore the efforts of many researchers are focused on an attempt to explain the mechanisms responsible for disease initiation and the maintenance of inflammation.

Nitric oxide (NO) is a small, very reactive molecule involved in many physiological processes, such as the regulation of smooth muscle tension, neurotransmission, and host defense. It plays important biological roles in the cardiovascular, respiratory, nervous, genitourinary, gastrointestinal, host-defense, hemopoietic, and musculoskeletal systems. *In vivo* it is synthesized from L-arginine by nitric oxide synthase (NOS). Three isoforms of NOS have been identified. Two of them (endothelial and neuronal, eNOS and nNOS) are constitutively expressed (cNOS) and are dependent on calcium-calmodulin complex concentration. The third is induced by some immunological conditions (iNOS) and is calcium-calmodulin complex independent. The expression of iNOS has been detected in various cells, including chondrocytes, synovial fibroblasts, osteoclasts, and macrophages. *In vitro* NO synthesis can be induced by proinflammatory cytokines such as IL-1 and TNFα and it is inhibited by glucocorticoids.

In animal models of collagen-induced arthritis, increased concentration of NO and increased activity of iNOS were observed in fibroblasts, synovial macrophages, and mononuclear leukocytes [1]. Spontaneous production of NO *in vitro* by synovial fibroblasts from RA and OA patients was described [2]. The present authors previously reported increased levels of NO metabolites (nitrite and nitrate) in the serum of patients with rheumatoid arthritis and osteoarthritis [3].

The aim of the present study was to evaluate the activity of iNOS in peripheral blood leukocytes of patients with early arthritis (EA) and established rheumatoid arthritis (RA) and to compare it with parameters of disease activity used in clinical practice.

Material and Methods

Thirty-five patients (28 females, 80%) who met the American College of Rheumatology (ACR) criteria for RA, 31 patients (20 females, 64.5%) with EA defined as arthritis of at least one joint lasting no longer than 12 months, and 30 healthy subjects (20 females, 66.7%) as a control group were enrolled in the study. Subjects with infections or a medical history revealing neoplasm were excluded from the study. All the patients with RA were treated with disease-modifying anti-rheumatic drugs (DMARDs), excluding TNF blockers. Some of them received glucocorticoids in a stable dose. The patients with EA were treated with sulfasalazine (SSZ) or received no DMARDs. The use of non-steroidal anti-inflammatory drugs was allowed in the RA and EA patients.

The activity of iNOS was measured by an immunocytochemical method in peripheral blood leucocytes separated by density gradient. The result was the percentage of stained cells per one hundred leukocytes. DAS 28 (Disease Activity Score 28), ESR (erythrocyte sedimentation rate), CRP (C-reactive protein), PLT (platelets), and the level of hemoglobin were also assessed.

The study was approved by the Silesian Piasts University of Medicine in Wrocław Bioethics Committee and all the subjects gave their written informed consent.

Results

The mean age of the RA patients was 55.7 (range: 27–74) years. Of the 35 RA patients, 25 (71%) had positive rheumatoid factor and erosions in the hands were found in 20 (57%). High disease activity (defined as DAS28>5.1) was detected in 18 patients and the mean DAS28 was 5.0 (range: 2.4–8.2). The mean disease duration was 13.0 (range: 139) years. The mean age of the EA patients was 44.1 (range: 18–77) years. Of the 31 EA patients, 10 (33%) had positive rheumatoid factor and no erosion in the hands was found. High disease activity was detected in 18 patients and the mean DAS28 was 5.2 (range: 1.4–8.1). The mean disease duration was 6.5 (range: 1–12) months. The mean age of the control group was 31.5 (range: 18–51) years. The patients' demographic and disease characteristics are shown in Tables 1 and 2. There was no statistically significant difference between BMI and disease activity between the RA and EA groups.

There was a statistically significant ($p < 0.05$) difference between RA and EA patients and healthy subjects in iNOS expression (25.4 ± 15.2%, 18.7 ± 12.3%, and 13.8 ± 15.1%, respectively) (Figure 1). No difference in iNOS expression between genders was found. Age and BMI seemed to have no impact on iNOS expression either. In EA, the difference in iNOS expression between subjects receiving SSZ and those receiving no DMARDs was statistically insignificant (15.9 ± 16.3% vs. 20.1 ± 10.2%, $p = 0.3$). In established RA, no significant impact of received DMARDs on iNOS expression was found, although in patients receiving MTX (methotrexat) and CyA (cyclosporine A), iNOS expression tended to be higher than in subjects receiving MTX with or without SSZ (32.5 ± 2.5% vs. 24.2 ± 16.5%, $p = 0.06$). In EA, mean iNOS expression was higher in patients with cigarette abuse than in nonsmokers (22.9% vs. 14.4%, $p < 0.05$), whereas no such a difference in established RA was observed.

In the RA and EA patients there was no difference in iNOS expression between RF-positive and RF-negative subjects. In the RA group, patients with erosive arthritis revealed significantly ($p < 0.05$) lower iNOS expression in peripheral blood leukocytes than those with non-erosive arthritis (23.3 ± 18.2% vs. 29.0 ± 7.3%, respectively).

In established RA, iNOS expression correlated negatively with CRP level, the number of tender and swollen joints, and DAS28. In EA, no correlation between iNOS expression and the analyzed parameters of disease activity was found (Table 3). In subjects with active RA (defined as DAS28 > 5.1), iNOS expression in peripheral blood leukocytes was significantly ($p < 0.05$) lower than in subjects with DAS28 ≤ 5.1 (18.7 ± 8.4% vs. 30.5 ± 17.3%, respectively). In the EA group the difference in iNOS expression between the subgroups with higher (DAS28 > 5.1) and lower (DAS28 ≤ 5.1) disease activity was not significant (17.8 ± 13.1% vs. 20.0 ± 11.6%); however, a similar tendency to that observed in RA was revealed.

Table 1. Demographic characteristics of the patients

Tabela 1. Charakterystyka demograficzna badanej populacji

	RA group (n = 35) (Chorzy na r.z.s.)	EA group (n = 31) (Chorzy na w.z.s.)
Females (%) (Kobiety)	28 (80%)	20 (64.5%)
Mean age – range (Średnia wieku – zakres)	55.7 (27–74)	44.1(18–77)
Mean BMI (*SD*) (Średnie BMI – *SD*)	26.8 (6.2)	26.6 (4.9)
Mean disease duration (range) (Średni czas trwania choroby – zakres)	13.0 (1–39) years	6.5 (1–12) months
Structure of employment (Struktura zatrudnienia) Employed – % (Czynni zawodowo – %) Unemployed because of health problems – %) (Niepracujący z powodów zdrowotnych – %)	 10 (28.6%) 10 (28.6%)	 14 (45.2%) 1 (3.2%)
Smoking – % (Palenie papierosów – %)	6 (17.1%)	16 (51.6%)

RA – rheumatoid arthritis, EA – early arthritis, BMI – body mass index.

R.z.s. – reumatoidalne zapalenie stawów, w.z.s. – wczesne zapalenie stawów, BMI – wskaźnik masy ciała.

Table 2. Characteristics of the disease in the study groups

Tabela 2. Charakterystyka choroby w badanych grupach

	RA group (n = 35) (Chorzy na r.z.s.)	EA group (n = 31) (Chorzy na w.z.s.)
RF (%)	25 (71%)	10 (33%)
Erosions on plain radiograms of hands or feet – % (Nadżerki na zdjęciach RTG rąk lub stóp – %)	20 (57%)	0
Number of tender joints – SD (Liczba bolesnych stawów – SD)	8.1 (5.4)	10.5 (10.6)
Number of swollen joints – SD) (Liczba obrzękniętych stawów – SD)	5.9 (4.8)	5.8 (5.1)
CRP (*SD*) [mg/dl] ESR (*SD*) [mm/h]	2.4 (2.8)	3.0 (4.4)
OB (*SD*) [mm/godz.] DAS28 (*SD*)	43.9 (24.7) 5.0 (1.37)	44.8 (29.9) 5.2 (1.56)
High disease activity (DAS28 > 5.1) – % (Wysoka aktywność choroby (DAS28 > 5,1) – %)	18 (51.4%)	18 (58.1%)
Hb (*SD*) [g/dl]	12.8 (1.2)	13.3 (1.7)
RBC [10^{12}/l] (*SD*)	4.1 (0.4)	4.2 (0.4)
WBC [10^{9}/l] (*SD*)	9.4 (3.5)	8.0 (2.7)
PLT [10^{9}/l] (*SD*)	267 (87)	255 (67)
Received DMARDs (Stosowane l.m.p.ch.)	26 – MTX +/– SSZ 3 – MTX + CyA 6 – other (inne)	10 – SSZ 21 – no DMARDs (bez l.m.p.ch.)

RF – rheumatoid factor, CRP – concentration of C-reactive protein, ESR – erythrocyte sedimentation rate, DAS 28 – Disease Activity Score 28, Hb – hemoglobin concentration, RBC – red blood cell count, WBC – white blood cell count, PLT – platelets, DMARDs – disease-modifying anti-rheumatic drugs, MTX – methotrexat, SSZ – sulfasalazine, CyA – cyclosporine A

RF – czynnik reumatoidalny, CRP – stężenie białka C-reaktywnego, OB – odczyn opadania krwinek Biernackiego, DAS 28 – wskaźnik aktywności choroby 28, Hb – stężenie hemoglobiny, RBC – liczba czerwonych krwinek, WBC – liczba białych krwinek, PLT – liczba płytek, LMPCh – leki modyfikujące przebieg choroby, MTX – metotreksat, SSZ – sulfasalazyna, CyA – cyklosporyna A

Discussion

This study demonstrated that iNOS expression in peripheral blood leukocytes was higher in patients with arthritis than in healthy subjects. Other authors also detected higher iNOS activity and elevated NO (nitric oxide) concentrations in synovial fluid and peripheral blood samples obtained from patients with RA than in healthy subjects [3–7]. NO production seems to be stimulated by various proinflammatory cytokines, for example IL-1 (interleukin-1), IL-17, and TNFα (tumor necrosis factor alpha) [8, 9] and it plays an important role in T-cell activation and development [10]. The role of NO in the pathogenesis of inflammatory arthritis is also supported by that fact that iNOS activity is suppressed by disease-modifying anti-rheumatic drugs and that this suppression is associated with diminished disease activity [11–14]. In the present study the authors did not detect any difference in the influence of DMARDs on iNOS expression.

A significant difference was also detected in the present study in iNOS expression in PBLs between RA and EA patients. Such a comparison was not described previously. Increased activation of iNOS in EA supports its role in the induction of the arthritis. Early activation of iNOS has been observed in animal models [15]. As the difference between iNOS expression in the PBLs of patients with RA and with EA cannot be attributed to the differences in disease activity (DAS28 score, ESR, and CRP level were similar in both groups), further observation is needed to determine whether higher iNOS expression in some patients with early arthritis can predict future RA development. Gonzalez-Gay et al. reported significant differences in the iNOS promoter polymorphism genotype frequency among northwest Spanish RA patients and they suggested that this may play an important role in susceptibility to RA [16]. The connection

Table 3. Correlation between iNOS expression and parameters of disease activity

Tabela 3. Korelacje między ekspresją iNOS a wskaźnikami aktywności choroby

	RA group (n = 35) (Chorzy na r.z.s.)	EA group (n = 31) (Chorzy na w.z.s.)
Number of tender joints (Liczba bolesnych stawów)	–0.295	–0.464*
Number of swollen joints (Liczba obrzękniętych stawów)	–0.222	–0.464*
CRP	–0.073	–0.392*
ESR OB	0.035	–0.340
DAS 28	0.018	–0.469*
Hb	0.053	0.137
PLT	–0.207	–0.009

* The correlation is statistically significant with $p < 0.05$.

* Korelacje istotne statystycznie; $p < 0,05$.

between iNOS expression and predisposition to certain a RA phenotype was also suggested by Heale et al. [17].

In the studied population, no difference in iNOS expression between genders was found. Age and BMI seemed to have no impact on iNOS expression either. In EA subjects, iNOS expression was higher in patients with cigarette abuse than in nonsmokers, whereas no such difference was observed in established RA. Wei et al. reported that cigarette smoking reduced iNOS activity in the lungs [18], but the influence of smoking on iNOS in peripheral blood was not examined. On the other hand, according to Krishnan et al., past smoking history was associated with increased risk for RA development [19]. Michou et al. found an association between cumulative dose of cigarette smoking and anti-CCP positivity [20]. The higher iNOS expression in smokers with EA detected in the present study needs further research aimed at answering the question whether smoking induces iNOS activity in PBLs and in this way promotes RA development. No association between iNOS expression and RF-positivity in RA and EA patients was found in this study.

In patients with RA, iNOS expression was higher in subjects with non-erosive arthritis than in subjects with erosive arthritis. In the RA group, negative correlation between iNOS expression and parameters of inflammation such as CRP level, number of tender and swollen joints, and DAS28 was also detected. What is more, iNOS expression in PBLs was significantly lower in subjects with active RA than in those with high disease activity. In the EA group the difference in iNOS expression between the subgroups with higher and lower disease activity was not significant, although a similar tendency to that observed in RA was revealed. The negative correlations between iNOS expression and parameters describing disease activity may be a consequence of the fact that NO seems to have divergent roles, being anti-inflammatory in chronic and proinflammatory in acute joint inflammation [21]. As RA is a chronic condition, the negative correlation between iNOS expression and disease activity supports the thesis of an anti-inflammatory property of NO in the chronic inflammatory processes. The different roles of NO may be a consequence of its heterogeneous influence on apoptosis. Some authors suggest that NO induces apoptosis in RA [22], but others report that NO prevents apoptosis by direct inhibition of cataspase-3 activation [23].

Although the sample size was limited in this study, its findings seem to add some new information to the knowledge about the role played by iNOS in arthritis. The differences that were detected between iNOS expression in healthy subjects

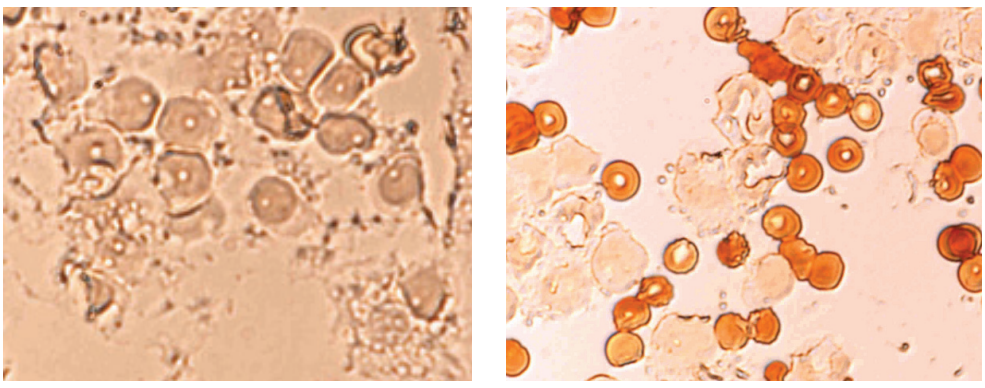

Fig. 1. iNOS expression in peripheral blood leukocyte: A – control group, B – RA subject

Ryc. 1. Ekspresja iNOS w leukocytach krwi obwodowej: A – grupa kontrolna, B – chory na r.z.s.

and in patients with RA were similar to those already reported, but the differences between EA and established RA were not described previously. As the difference between iNOS expression in PBLs of patients with RA and with EA cannot be attributed to differences in disease activity (DAS28 score, ESR, and CRP level were similar in both groups), further observation is needed to determine whether higher iNOS expression in some patients with early arthritis can predict future RA development. The sample size is too small to study the interaction between iNOS activity and environmental factors such as tobacco smoking and their influence on RA development. Further studies with a larger sample size of unselected patients with EA and RA is required before definite conclusions can be drawn.

In conclusion, these findings suggest an association between iNOS expression and different forms of arthritis. They also suggest an association between iNOS and cigarette smoke in EA.

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