ORIGINAL ARTICLE

Nuclear factor-erythroid 2-related 2 mRNA and protein are highly expressed in the synovium of patients with rheumatoid arthritis

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Abstract

Introduction: Nuclear factor-erythroid 2-related factor 2 (Nrf2) is the main transcription factor for antioxidant stress response. The aim of this study was to analyze the expression level of Nrf2 mRNA and protein in the synovium of patients with rheumatoid arthritis (RA), and the association between Nrf2 mRNA and oxidative stress.

Materials and Methods: The synovia harvested from 41 patients with RA served as the study group and 4 cases with knee osteoarthritis (OA) were registered as the control group. The Nrf2 mRNA expression level was evaluated using real-time polymerase chain reaction (PCR) and the Nrf2 protein via immunohistochemistry. The immunopositivity of the Nrf2 protein was then graded into four stages (none, slight, moderate, and strong) during microscopic examination. In addition, d-reactive oxygen metabolites (d-ROM) measurement was performed using the sera of patients with RA.

Results: The expression level of Nrf2 mRNA in the RA synovia (2.34±0.86) was significantly higher than that in the OA synovia (1.5±0.49). The OA synovia had neither moderate nor strong intensity of Nrf2 protein immunopositivity. In the RA synovia, moderate and strong intensities were noted in 23 and three cases, respectively. The immunoreactivity of the Nrf2 protein in the RA synovia was significantly stronger than that in the OA synovia. The Nrf2 mRNA showed significantly correlation with preoperative d-ROM.

Conclusion: The present study demonstrated increased upregulation of Nrf2 in the RA synovia compared to that in the OA synovia both at the mRNA expression and protein levels. An increased expression of Nrf2 mRNA may reflect an upregulation of the antioxidant capacity in response to high oxidative stresses.

Keywords: Rheumatoid arthritis, NF-E2 related factor 2 (Nrf2), synovium, reactive oxygen metabolites (ROM), biological antioxidant potential (BAP)

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease typically characterized by progressive joint damage. Oxidative stress is associated with the pathogenesis of RA.[1] Many oxidative markers such as oxygen-derived radicals (superoxide radical, peroxyl radical, perhydroxyl radical, and hydroxyl radical) and non-free radical species (hydrogen peroxide and singlet oxygen) have been analyzed in patients with RA.[2] Our previously studies have demonstrated that reactive oxygen species are upregulated, and biologics such as etanercept and tocilizumab prominently downregulated the oxidative stress in patients with RA.[3-5]

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a key transcription factor for antioxidant stress response. It belongs to the cap 'n' collar subfamily containing the basic leucine zipper region and binds to the antioxidant response elements (AREs) located in the promoter regions.[6] Studies conducted in Nrf2-deficient, collagen-induced arthritis

(CIA) mice models demonstrated the occurrence of more severe arthritis. These findings suggested that Nrf2 plays a pivotal role in redox homeostasis during exposure to oxidative stress and in the regulation of the severity of RA.[7, 8]

The association between increased Nrf2 expression level in the RA synovium and disease activity, oxidative stress, and antioxidant potential in patients with RA has not been clearly elucidated.

The present study was intended to evaluate the expression level of Nrf2 mRNA and protein in the RA synovium, and the association between Nrf2 mRNA and some inflammatory markers, oxidative stress, and antioxidant potential.

Patients and methods

The synovia were harvested from 41 RA patients who fulfilled the 1987 ACR classification criteria, recruited between July 2010 and May 2012. The synovia were harvested during joint surgeries such as synovectomy and arthroplasty.[9] The patients with RA were divided into two groups: conventional synthetic disease modifying anti-rheumatic diseases (csDMARDs) group (32 cases) and biological disease modifying anti-rheumatic diseases (bDMARDs) group (9 cases). In the bDMARDs RA group, etanercept was used in six cases, and infliximab, adalimumab, and tocilizumab in one case each.

Four patients with knee osteoarthritis (OA) (average age, 75.5±4.4 years) were included as the control group, and their synovia were harvested during the surgery (total knee arthroplasty). Demographic data of all the subjects are shown in table 1. The most commonly operated surgical site in patients with RA was the knee joints (17 cases) (Table 2). Informed consents were obtained from all the recruited

Table 1: Demographic data of the recruited subjects

Variables	OA	RA-csDMARDs	RA-bDMARDs	P value
Cases	4	32	9	-
Age (years)	75.5±4.4	65.8±10.3	64±13	-
Sex (M/F)	1/3	5/27	1/8	-
RA duration (years)	-	17.4±11.4	10.5±5.8	0.087
Steinbrocker class (2/3/4)	-	16/13/3	2/7/0	-
Steinbrocker stage (II/III/IV)	-	1/20/11	0/9/0	-
Pre-operative CRP (mg/dl)	-	2.2±3.6	2.0±2.6	0.84
Pre-operative ESR (mm/hr)	-	50±33	69±34	0.141
MTX (mg/week)	-	4.4±3.7	2.7±4.1	0.225
MTX (%)	-	63	33	0.147
PSL (mg/day)	-	2.6±3.5	4.7±3.8	0.114
PSL(%)	-	50	67	0.466
DAS28	-	4.4±1.1	4.9±0.8	0.322

Table 2: Distribution of various surgical sites among the RA patients

Surgical sites	RA-csDMARDs	RA-bDMARDs	Total
Knee	13	4	17
Wrist	10	1	11
Elbow	6	1	7
Hip	3	1	4
Shoulder	0	2	2
Total	32	9	41

subjects before surgery. Ethics Committee approval was obtained in advance to the study from Niigata University Medical and Dental General Hospital (Code#1345).

Tissue preparation

Synovia harvested during the surgeries were kept immersed in 10% formalin for 3 days. Thereafter, each specimen was dehydrated with a graded series of ethanol, prior to being embedded in paraffin. Paraffin sections with 4 μm thickness were cut using a microtome (Leica, Tokyo, Japan) and stored at 4°C for the immunohistochemistry analysis of the Nrf2 protein.

Quantitative real-time polymerase chain reaction (PCR) analysis of the Nrf2 mRNA

Total RNA was extracted from each specimen (synovium) via a guanidium thiocyanate-phenol-choloroform extraction (ISOGEN) (Nippon Gene, Tokyo, Japan). The yield and purity of the RNA were determined on the basis of the spectrophotometric measurements of the ratio of UV absorbance at 260 nm and 280 nm. The first strand cDNA was synthesized from the total RNA using the PrimeScript RT Reagent Kit (TaKaRa Bio, Shiga, Japan). A quantitative real-time PCR analysis was performed using SYBR Premix EX Taq II (Tli RNaseH Plus; TaKaRa, Shiga, Japan), and the results were analyzed using the Thermal Cycler Dice Real Time System TP800 (TaKaRa, Shiga, Japan).

The primer sequences used were as follows: primer pairs used for human nuclear factor, erythroid 2-like 2 (NFE2L2), 5′-AGCCCAGCACATCCAGTCAG -3′ (forward) and 5′-TGCATGCAGTCAAAGTACAAAG-3′ (reverse); glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′- GCACCGTCAAGGCTGAGAAC-3′ (forward) and 5′-TGGTGAAGACGCCAGTGGA-3′ (reverse). The gene copy numbers for Nrf2 were calculated using a standard curve. Moreover, quantitative mRNA expression levels were calculated by normalizing them against one synovium isolated from a patient with OA. Same amount of RNA was applied to the normalization by using G3PDH.

Immunohistochemistry analysis of the Nrf2 protein

Sectioned specimens on the slide glass were deparaffinized in xylene and rehydrated with a graded series of ethanol to 70%. The antigen retrieval with 0.1% trypsin at 37°C for 20 minutes was performed. The tissue sections were treated with 0.3% hydrogen peroxide in methanol for 30 minutes to inhibit endogenous peroxidase and then incubated with 10% normal goat serum to reduce non-specific reactions. The sections were incubated with anti-Nrf2 antibody (ab53019) (abcam, Tokyo, Japan) diluted 1:100 with 10% normal goat serum for 16 hours at 4°C and subsequently with the second antibody, Histofine MAX-PO (MULTI) (Nichirei, Tokyo, Japan. The peroxidase reaction products were visualized using 3'-diaminobenzidine tetrahydrochloride (Nichirei, Tokyo, Japan). The sections were counterstained with hematoxylin.

Evaluation of the Nrf2 protein immunoreactivity

Based on the immunohistochemical staining of the Nrf2 protein under light microscope examination, the percentage of Nrf2-positive cells was evaluated.[10] According to the percentage of reactive cells, the immunostaining was graded on a sliding scale of 0 to 3+ as follows: 0: no staining; 1+: 0-10% cells stained with weak intensity; 2+: 10-40% cells stained with variable intensity; 3+: >40% of cells stained with strong intensity. In this study, the degree of immunopositivity was defined and divided into four grades ('none' for 0, 'slight' for 1+, 'moderate' for 2+, and 'strong' for 3+).

For the comparison of OA with RA data, the immunopositivity findings of the Nrf2 protein were rearranged on a 2×2 contingency table by subgrouping as 'none/slight' and 'moderate/strong'.

Measurements of the serum oxidative and antioxidative potentials

Serum samples were collected just before the joint surgery and was subjected to d-reactive oxygen metabolites (d-ROM) and biological antioxidant potential (BAP) tests to evaluate the oxidative and antioxidant capacities using the Free Radical Elective Evaluator (FREE) (Diacron, Italy). Sera had been stored at -80°C (deep freezer) just before the tests. The time interval between serum collection and the tests was not more than 1 month.

For the d-ROM measurement, a 20-μL serum sample was added to 1 mL of buffered solution (R2 reagent kit), and 20 μL of chromogenic substrate (R1 reagent kit) was added to the cuvette. After mixing, the sample was immediately incubated in the thermostatic block of the analyzer for 5 minutes at 37°C. Absorbance was recorded at 505

nm. The measurement was recorded as Carratelli units (U.CARR, one U.CARR corresponds to 0.08 mg/dL H_2O_2). The reference value suggested by the manufacturer was <300 U.CARR. The values were defined for the d-ROM; values higher than 300 U.CARR suggest a high oxidative stress.[5]

For the BAP test, the serum was added to a colored solution obtained by mixing a ferric chloride solution with a thiocyanate derivative solution, which causes decolorization. The intensity of decolorization was measured photometrically at 505 nm, which was proportional to the antioxidant capacity.[11] The normal reference value for the BAP test results was <2200 μmol/L, which suggests a low antioxidant potential.

Statistical analysis

The graph pad prism 6J (Tokyo, Japan) was used for the statistical analysis. Results with P values <0.05 were considered as statistically significant. Mann-Whitney U test was used for comparing the Nrf2 mRNA levels between the patients with OA and RA. The Fisher's exact test was used for comparing the Nrf2 protein immunopositivity between OA and RA. The Pearson's correlation coefficient test was performed to determine the correlation between Nrf2 mRNA expression level and disease activity score 28 (DAS28), CRP, ESR, BAP, and d-ROM in patients with RA.

Results

Demographic data

No significant difference was found between the csDMARDs and bDMARDs RA groups with respect to age, RA disease duration, preoperative c-reactive protein (CRP) level, preoperative erythrocyte sedimentation rate (ESR), methotrexate (MTX) dose, MTX treatment percentage, prednisolone (PSL) dose, PSL treatment percentage, and DAS28 (Table 1).

Expression level of the Nrf2 mRNA from the synovia

The expression level of the Nrf2 mRNA in the OA (n=4) and RA synovia (n=41) were 1.5±0.49 and 2.34±0.86 respectively. A significant difference was noted between the OA and RA synovia (Mann-Whitney U test, P=0.041). The Nrf2 mRNA level was 2.26±0.90 in the csDMARDs RA group and 2.63±0.66 in the bDMARDs RA group, and no significant difference was detected between the 2 groups (unpaired t-test, P=0.36). In addition, a significant difference was found between the OA and bDMARDs RA groups (Mann-Whitney U test, P=0.020; Fig. 1).

Immunohistochemistry analysis of the Nrf2 protein

The Nrf2 protein was strongly expressed in the RA synovium compared to that in the OA synovium. In particular, Nrf2 protein-immunopositive cells were observed in the superficial synovial cells and pericytes of

Fig. 1: Expression level of Nrf2 mRNA in OA and RA synovia

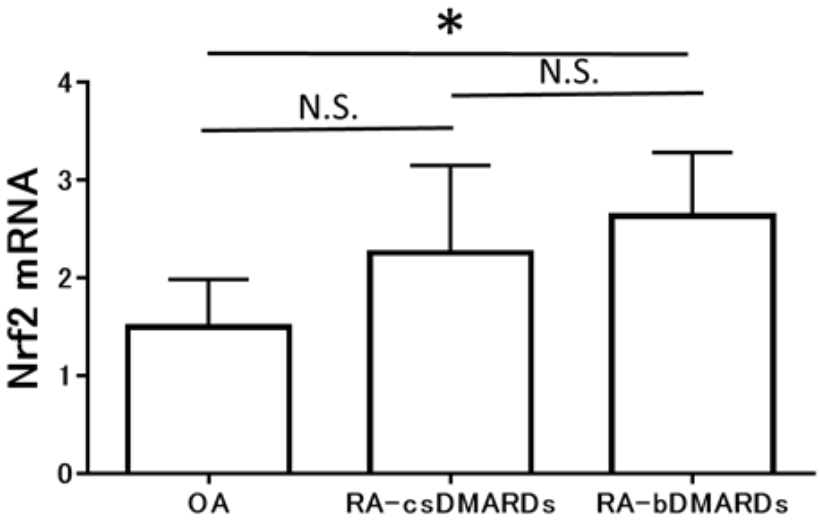

Fig. 2A: Immunohistochemical findings of Nrf2 protein in OA synovium

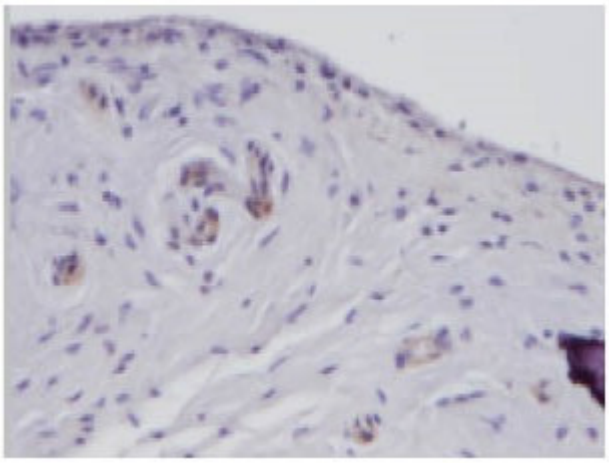

Fig. 2B: Immunohistochemical findings of Nrf2 protein in RA synovium

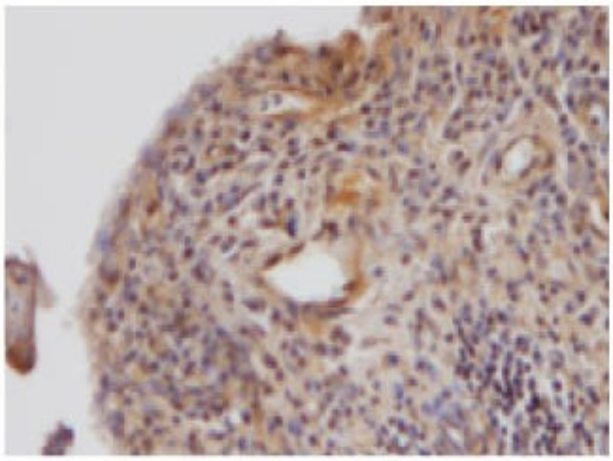

Table 3A: Comparison of Nrf2 protein immunopositivity between OA and RA groups

Degree of Nrf2 protein	OA	RA	Total
None	2	4	6
Slight	2	11	13
Moderate	0	23	23
Strong	0	3	3
Total	4	41	45

Table 3B: Comparison of the significance of Nrf2 protein immunopositivity between the OA and RA groups at various levels

Degree of Nrf2 protein	OA	RA*	Total
None/ Slight	4	15	19
Moderate/ Strong	0	26	26

* Fisher's exact test, P <0.01, Nrf2: nuclear factor-erythroid 2-related factor 2; OA: osteoarthritis; RA: rheumatoid arthritis

the vessels in the RA synovium (Fig. 2A and 2B). The OA synovia had neither moderate nor strong intensity of Nrf2 protein immunopositivity. In the RA synovia, 23 cases had moderate intensity and three had a strong intensity; four had no intensity and 11 had a slight intensity (Table 3A).

The statistical analysis showed that the immunoreactivity of the Nrf2 protein in the RA synovia was significantly stronger than that in the OA synovia (Fisher's exact test, P<0.01) (Table 3B).

Oxidative stress and antioxidative stress status in the patients with RA

The average d-ROM value was 512±170 (range, 219-926) U.CARR. The average BAP value was 2979±1068 (1468-6188) µmol/L.

Table 4: Correlation of various parameters with Nrf2 mRNA expression level in RA patients

Parameters	Pearson's correlation coefficient	P value
DAS28	0.26	0.13
ESR	0.04	0.85
CRP	0.26	0.10
BAP	0.09	0.56
d-ROM	0.52	0.01*

*Statistically significant, DAS28: disease activity score 28; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; BAP: biological antioxidant potential; d-ROM: d-reactive oxygen metabolites; Nrf2: nuclear factor-erythroid 2-related factor 2; RA: rheumatoid arthritis

Correlation between Nrf2 mRNA expression level and inflammatory markers, d-ROM, and BAP in the patients with RA

The Nrf2 mRNA was significantly correlated with the preoperative d-ROM value (r=0.52, P=0.01). However, there was no significant correlation between the Nrf2 mRNA and DAS28 (r=0.26, P=0.13), ESR (r=0.26, P=0.1), CRP (r=0.09, P=0.56), and BAP (r=0.04, P=0.85) (Table 4).

Discussion

Nrf2 is a redox-sensitive transcription factor that binds to the ARE located in the promoter regions of detoxifying/antioxidant genes.[11] However, it remains unclear whether the Nrf2 mRNA expression level or Nrf2 production degree is high in patients with RA. Additionally, the parameters that are significantly correlated with the degree of Nrf2 expression in clinical samples has not been identified.

In the current study, the Nrf2 mRNA expression levels were found to be significantly higher in the RA synovia than in the OA synovia, suggesting that the antioxidant potential was more upregulated in RA than in OA. However, no significant difference was detected between csDMARDs (2.26±0.90) and bDMARDs (2.63±0.66) in Nrf2 mRNA (P=0.36, unpaired t-test). Based on the findings, it has been concluded that bDMARDs was not a responsible factor for increasing Nrf2 mRNA. Only 9 cases were considered in bDMARD group, hence no significant difference was found between TNF inhibitor and non-TNF inhibitors. RA patients in the present study were refractory to DMARDs and required one of the joint surgeries such as arthrodesis, arthroplasty, or synovectomy for treating persisted inflamed joints.

In addition, the production of Nrf2 protein was found to be more significant in the RA synovia than in the OA synovia. This finding was consistent with study conducted by Wruck *et al.* in Nrf2-knockout mice.[7] Very few studies have investigated the Nrf2 mRNA and protein expression level in the RA synovia compared OA synovia in clinical settings. Thus, the current study data would be useful for verifying the degree of antioxidant potentials in the patients with RA.

An *in vitro* study demonstrated that cilostazol, an inhibitor of phosphodiesterase type III showing a potent anti-inflammatory effect, upregulates heme oxygenase 1-gene via the Nrf2 signaling pathway and enhances apoptosis in RA fibroblast-like cells.[12] In the chondrocytes isolated from patients with OA, sesamin showed anti-inflammatory effects in interleukin (IL)-1β-stimulated chondrocytes by activating the Nrf2 signaling pathway.[13]

In the RA group, the average d-ROM value (512 U.CARR) noted was higher than the reference value (300 U.CARR). This finding indicates that patients with RA requiring joint surgeries due to synovitis or joint destruction had higher oxidative stress. The disease activity score (DAS28) in these patients was 4.5±1.1 on average, suggesting moderate disease activity. In one of our previous studies, the averaged-ROM value noted at the initiation of tocilizumab administration in 11 patients with RA was 392±110 U.CARR, indicating a higher oxidative stress.[5]

Conversely, the BAP value was 2979±1068 μmol/L in this study, which was found to be higher than the reference value. The normal reference interval of BAP test results determined as the mean±standard deviations (SDs) of values obtained from the 312 healthy volunteers was

2541±122 μmol/L.[11]

Higher antioxidant potentials suggest their upregulation in response to high oxidative stress in patients with RA. However, the Nrf2 mRNA expression level was not significantly correlated with DAS28, CRP, ESR, and BAP levels, suggesting that local Nrf2 mRNA expression does not reflect systemic antioxidant potentials. The reason for this discrepancy remains unknown. In addition, the Nrf2 mRNA expression significantly correlated with the d-ROM value; thus, indicating the increased upregulation of Nrf2 mRNA in the operated joints in response to higher preoperative oxidative stresses.

Most of the studies on oxidant potentials have demonstrated that the total reactive oxygen species (ROS), hydrogen peroxide, superoxide radical, and hydroxyl radical levels were higher in patients with RA.[2] Conversely, the activity of antioxidant enzymes in patients with RA are controversial. There are various reports that antioxidant levels in patients with RA are increased, decreased, and even equal compared with those of the control subjects.[2] Staron *et al.* demonstrated that the antioxidant enzyme activity levels of superoxide dismutase (SOD), glutathione (GSH) and -SH was lower in patients with RA.[14]

García-González *et al.* reported that oxidative damage was elevated in patients with RA, and SOD and GSH levels and GSH/GSSG ratio were also higher in these patients than in the control group, and these upregulations were insufficient to prevent oxidative damages. These findings were in agreement with the present study data showing elevated oxidative and antioxidant status.[15]

The limitation of the present study is the small sample size, especially in the OA control group (only four cases). In addition, the mechanism by which oxidative stress directly or indirectly upregulates Nrf2 mRNA expression level in operated swollen or destructive joints has not been clearly elucidated. Mammalian target of rapamycin (mTOR) plays a crucial role in cell survival and is a key regulator of autophagy. In human THP-1 monocytes, inflammation by ROS was remarkably inhibited by sonodynamic therapy via PI3K/Akt/mTOR signaling pathway.[16] Dietary restriction or rapamycin treatment decreased mTOR signaling pathway and resulted in downregulation of ROS within mitochondria in mouse brain.[17] mTOR pathway is one of the related signaling pathways in RA pathogenesis.[18] So the mTOR pathway and mitochondrial dysfunction (accumulation of ROS) may be implicated in RA pathogenesis and further research is mandatory to investigate the associations.

The present study findings suggest that Nrf2 upregulation may be just a bystander activation in response to upregulation of ROS in RA pathogenesis. Further evaluation of the detailed mechanism is essential for understanding the association between Nrf2-Keap1 signaling pathway and RA pathogenesis.

Conclusion

In summary, the study has demonstrated the increased upregulation of antioxidant stress response transcription factor Nrf2 in the RA synovia compared to OA synovia at the mRNA expression and protein levels. The Nrf2 mRNA levels were significantly correlated with the preoperative d-ROM value, suggesting that the higher expression of Nrf2 mRNA may reflect an upregulation of the antioxidant capacity in response to high oxidative stresses in RA patients.

Competing interests

The authors declare that they have no competing interests.

Citation

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