Original Article

Relationship between P-glycoprotein and multidrug resistance in refractory regulatory mechanism and influence of TNF-α on the P-glycoprotein

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Received August 23, 2015; Accepted December 9, 2015; Epub February 15, 2016; Published February 29, 2016

Abstract: To elucidate the clinical relevance of P-gp expression and function to MDR in patients with rheumatoid arthritis (RA). Samples were divided into 4 groups: the control, incipient RA, effectively cured RA, refractory RA group. First, P-gp expression was determined by FCM, P-gp function by Rh-123 accumulation assay, mRNA expression of P-gp in PBMC by RT-PCR and TNF-α level in serum by ELISA. Correlation analysis between them was performed. Then, interference by TNF-α on PBMC from the 4 groups for 2, 6, 12 and 24 h was performed, respectively. P-gp expression and function was determined by FCM and Rh-123 accumulation assay, respectively. P-gp expression and function, TNF-α mRNA expression and serum TNF-α level in the control were all much lower than that in RA groups. In RA groups, the values in refractory RA group were much higher than the other groups, and values in effectively cured RA group were the lowest. P-gp expression had a positive correlation with mRNA expression of TNF-α, serum TNF-α level and DAS28 score. However, the results on Rh-123 fluorescence intensity were opposite. After interference by TNF-α, P-gp expression and function in refractory RA group were sensitive to the stimulation of TNF-α with same concentration in RA groups. TNF-α can up-regulate PBMC P-gp expression and function in patients with RA, mediate the MDR of RA and participate in the formation of RRA.

Keywords: Rheumatoid arthritis, TNF-α, P-gp, PBMC, multidrug resistance

Introduction

Rheumatoid arthritis (RA) is the most common systemic autoimmune disease, affecting 1% of the adult population worldwide, with an estimated heritability of 60% [1]. Treatment of RA may differ among rheumatologists and currently, clear and consensual international recommendations on RA treatment are not available [2]. The treatment for RA mainly includes non-steroidal anti-inflammatory drugs (NSAIDs), disease modifying anti-rheumatic drug (DMARDs) and biological agent.

In clinic, although many patients were treated with multiple DMARDs, the curative effect for partial patients was poor [3]. Moreover, the curative effect decreases gradually with the cure time increases, which result in the drug resistance and failure of treatment. One of the major problems in chemotherapy is multidrug resistance (MDR) against anticancer drugs [4]. MDR is a phenomenon whereby human tumours that acquire resistance to one type of therapy are found to be resistant to several other drugs that are often quite different in both structure and mode of action [5]. It has gotten enough attention in the treatment for tumors and infectious diseases, but has received little attention in RA. The mechanism of MDR is complicated and mediated by poly-molecular and multiple ways. From the perspective of pharmacodynamics, MDR involves the transmit damage from drug to target cells, disorders of drug uptake, increase of drugs discharge, changes of drug activity in cells, and inhibition of drug target [6]. Among them, the increase of drugs discharge mediated by transmembrane trans-
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P-glycoprotein (P-gp) is a member of the ATP-binding cassette transporter family and the product of the MDR1 gene [7]. It is a transmembrane efflux pump for different lipophilic compounds, including many anticancer drugs and fluorescent dyes [8]. It is able to confer resistance on tumors against a large number of functionally and chemically distinct cytotoxic compounds. Study showed the overexpression of P-gp on the surface of tumor cells causes MDR [9]. Significant levels of P-glycoprotein have been detected in a number of human tumors. The overexpression of P-gp can discharge antineoplastic drugs from intracellular to extracellular resulting in the decrease of drug content and reaction to drugs, and lead to MDR [10]. Tumour Necrosis Factor α (TNF-α), is an inflammatory cytokine produced by macrophages/monocytes during acute inflammation and is responsible for a diverse range of signaling events within cells, leading to necrosis or apoptosis [11]. Its therapies represent an important advancement in therapy for RA [12]. It is a critical mediator of inflammation and metabolic response in patients with RA [13]. Researched show TNF-α participate in the regulation of P-gp and formation of MDR [14]. However, there are no reports about whether TNF-α participate in the regulation of P-gp and the mechanism.

In this study, we aimed to study the influence of TNF-α on the regulation of P-gp and clarify its effect on RA MDR in molecular levels.

Materials and methods

Patients

The subjects were 60 patients (Rheumatism Immunity Branch of the Second Hospital of Xiangya, Central South University) with RA who met the diagnostic criteria of the American College of Rheumatology and 20 normal volunteers. In addition, the study included peripheral blood samples from 20 healthy adults, matched by age and sex to the patients with RA. The control subjects were selected in Voluntary Blood Donation Vehicle. They were divided into 4 groups: incipient RA, effectively cured RA, refractory RA group and the normal control (NC). Each group had 20 samples. All RA patients were performed laboratory examination and Disease Activity Score (DAS28). Gender and age of patients in the 3 RA groups had no significant difference. For incipient RA group, patients were first visiting to hospitalization, accorded with RA diagnostic criteria and performed no treatment. For effectively cured RA group, methotrexate and leflunomide were used for RA patients and the treatment reached to criteria of the American Rheumatism Association for rheumatoid arthritis ACR20. For refractory RA group, patients were treated with NSAIDs, hormone with small dose (20 mg/d) or the unite application of methotrexate and leflunomide, but the treatment was ineffective.

Collection of peripheral blood mononuclear cells and serum separation

Elbow venous blood, procoagulant (2 mL) and heparin anticoagulation (20 mL) were collected at early morning. Procoagulant was centrifuged at 3000 r/min for 10 min to obtain serum. Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation. Heparin anticoagulation of 10 mL was mixed with the equal RPMI1640 medium (Gibco, The Dexter Corporation, Ohio, USA), transferred to lymphocyte separation medium and centrifuged at 2000 r/min for 20 min. The mixture was divided into three layers: the upper layer was plasma and RPMI1640 and the middle layer was mononuclear cell. The mononuclear cell was collected and five times the volume of RPMI1640 was added. Centrifugation at 1500 r/min for 15 min was performed and PBMC. Trypan blue (Beijing Dingguochangsheng biotechnology co., LTD, Beijing, China) staining was used for cell count and observed with microscope (Nikon80i; Nikon, Tokyo, Japan). Living cells were not stained and bright. For the dead cells, their color was irregularly and the shapes were different.

Function determination of PBMC P-gp

Separated PBMC was suspended into RPM-1640 medium containing 5 μg/ml Rhodamine 123 (Rh123) (Sigma, St Louis, USA) again to 1×10⁶/mL, and cultivated in a humidified cell incubator with an atmosphere of 5% CO₂ at 37°C. The cell suspension was then centrifuged at 1500 r/min for 5 min and washed with PBS for two times. Cells were resuspended to 1 ml pre-cooled PBS and flow cytometry (FCM) (FACSCalibur; BD Biosciences, USA) was used to detect the fluorescence intensity of Rh123 (λex =488 nm, λem =525 nm).
mRNA expression of TNF-α by RT-PCR

RT-PCR was performed to evaluate the mRNA expression of TNF-α. Total RNA was isolated with an RNA isolation kit (Takara Shuzo, Kyoto, Japan), according to the manufacturer’s protocol, and the RNA quality was confirmed by an optical density measurement of A260/A280 >1.8. PCR product formation was monitored continuously using Sequence Detection System software, version 1.7 (Applied Biosystems, Foster City, CA, USA). The cDNA was amplified by 25 PCR cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 45 s, using Taq DNA polymerase and the following primers: 5'-CAGAGGGAGAAGTCCCATCAT-3' and 5'-CTTGGCTCTAGAGACG-3' for TNF-α, and 5'-CACGATGGAGGCTCATC-3' and 5'-TAAAGACCTGACTGACCCAC-3' for β-actin. The PCR products were electrophoresed in 1.5% agarose gels, visualized by ethidium bromide staining, and photographed under ultraviolet light. The primers for real-time PCR were the same as those for RT-PCR. Accumulated PCR products were detected directly by monitoring the increase in the reporter dye (SYBR) Green. The quantity of each transcript was calculated as described in the instrument manual and normalized to the amount of β-actin.

TNF-α level by ELISA

TNF-α was determined in the uterine secretions, serum and plasma using a commercially available ultra sensitive ELISA kit (Laboserv Diagnostica, Giessen, Germany). Following centrifugation the supernatants of the samples were analyzed. The ELISA was performed according to the instructions of the manufacturer. The serum and plasma concentration of TNF-α was measured to estimate the contamination of the samples with TNF-α from blood. The TNF-α concentration in the samples was expressed relative to the sample’s protein content, determined according to Lowry et al.

P-gp expression and function of PBMC after intervention by TNF-α

The isolated PBMC was resuspended into RPMI-1640 medium containing 10% PBS to 1×10^7/mL. Each sample was set the control and intervention by TNF-α for 2, 6, 12 and 24 h. For the TNF-α intervention groups, samples were placed on 24-well plate with 300 μL/well. RPMI-1640 medium containing 10% PBS was added to the well to 1 ml/well and TNF-α content were adjusted to 0.5 ng/ml. The plates were cultivated in a humidified cell incubator with an atmosphere of 5% CO₂ at 37°C for 2, 6, 12 and 24 h, respectively. Cells were collected, and FCM and Rh123 were used to detect the expression and function of PBMC P-gp, respectively.

Statistical analysis

Results were expressed as mean ± SD. Data analysis was made by analysis of variance (ANOVA) followed by LSD-t post-hoc test for multiple comparisons using the computer statistical package SPSS 18.0. A paired Student’s t-test was used to evaluate differences among normal, Incipient, Effectively and Refractory group. Multiple group comparisons were analyzed using ANOVA with a post hoc test. P<0.05 was considered to indicate a statistically significant result.

Results

Relation between P-gp, MDR on RA and TNF-α

PBMC P-gp expression: Survival rate of all separated PBMC was determined and results showed the cell survival rate in every group was above 95%. P-gp expressions of PBMC were determined by FCM and results were shown in Figure 1A. As shown, P-gp expression of PBMC in the normal was low and the difference had statistical significance compared with incipient RA, effectively cured RA and refractory RA group (P<0.01). P-gp expression in refractory RA group was significantly higher than incipient RA, and effectively cured RA group (P<0.01). P-gp expression in incipient RA group was much higher than effectively cured RA group (P<0.05).

Function of P-gp in PBMC: The function of P-gp can be reflected by the accumulation of Rh123 in cells. The stronger the Rh123 fluorescence intensity in cells, the weaker the P-gp function was. On the contrary, the weaker the Rh123 fluorescence intensity in cells, the stronger the P-gp function was. In this study, fluorescence intensity in the control was significantly lower than the other groups and the difference had statistical significance (P<0.01), which stated function of P-gp in the control was weakest.
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Fluorescence intensity in refractory RA group was lower than incipient and effectively cured RA group (P<0.01). Fluorescence intensity in effectively cured RA group was significantly higher than the incipient RA group (P<0.05) (Figure 1B). Those indicated in RA groups, the function of P-gp in refractory RA group was the strongest and in effectively cured RA group was the weakest.

mRNA expression of TNF-α in PBMC: As determined by Ultraviolet Spectrometry Photometer, the A260/280 value of extracted RNA was above 1.8 and was accordance with the inspection requirement of RT-PCR. The PCR product size of TNF-α mRNA after amplification by RT-PCR was consistent with the anticipation. The results of RT-PCR showed the mRNA expression of TNF-α in the control was notable lower than RA groups' (P<0.01) and the differences had significant difference (P<0.01). Among them, mRNA expression of TNF-α in refractory RA group was the highest and the lowest was occurred in effectively cured RA group (Figure 1C).

TNF-α level in serum by ELISA: Serum TNF-α level in every group was determined by ELISA method and results were shown in Figure 1D. As shown, TNF-α level in RA groups was much higher than the control (P<0.01). In refractory RA group, TNF-α level was notlly higher than incipient and effectively cured RA group (P<0.01) and the level in incipient group was higher than effectively cured group (P<0.01).

DAS score in every RA group: Disease activity DAS score was performed in RA groups. As shown, DAS28 score in incipient RA group was 5.70±1.46 which was markedly higher than effectively cured group (3.78±0.79, P<0.01). The value in refractory RA group was 7.02±0.93 which was significantly higher than the other two groups (P<0.01).

Correlation analysis: The Correlation analysis results showed there was a positive correlation between P-gp expression in PBMC and serum TNF-α level (r=0.758, P<0.01), and a negative correlation between Rh123 fluorescence intensity and serum TNF-α level (r=-0.863, P<0.01). mRNA expression of TNF-α in PBMC had a posi-
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Figure 2. Influence of TNF-α on P-gp expression and Rh-123 fluorescence intensity in the normal group. A: P-gp expression after TNF-α intervention for 0, 2, 6, 12 and 24 h; B: Rh-123 fluorescence intensity after TNF-α intervention for 0, 2, 6, 12 and 24 h; *P<0.05, compared with 0 h, the difference has statistical significance; **P<0.01, compared with 0 h, the difference has significant statistical significance; "P<0.05, compared with 2 h, the difference has statistical significance; ""P<0.01, compared with 2 h, the difference has significant statistical significance; &P<0.05, compared with 6 h, the difference has significant statistical significance.

Figure 3. Influence of TNF-α on P-gp expression and Rh-123 fluorescence intensity in incipient RA group. A: P-gp expression after TNF-α intervention for 0, 2, 6, 12 and 24 h; B: Rh-123 fluorescence intensity after TNF-α intervention for 0, 2, 6, 12 and 24 h; *P<0.05, compared with 0 h, the difference has statistical significance; **P<0.01, compared with 0 h, the difference has significant statistical significance; #P<0.05, compared with 2 h, the difference has statistical significance; ##P<0.01, compared with 2 h, the difference has significant statistical significance; &P<0.05, compared with 6 h, the difference has significant statistical significance; &&P<0.01, compared with 6 h, the difference has significant statistical significance.

tive correlation with P-gp expression in PBMC (r=0.29, P<0.01) and negative correlation with Rh123 fluorescence intensity (r=-0.843, P<0.01). As for P-gp expression and DAS28, there was positive correlation between them (r=0.588, P<0.01), and a negative correlation between Rh123 fluorescence intensity and DAS28 (r=-0.702, P<0.01).

TNF-α intervention

Effect of TNF-α on PBMC P-gp expression and function in the control: In the control group, P-gp expression after TNF-α intervention for 2 h had no statistical difference with pre-intervention, and the value after TNF-α intervention for 6 h was significantly higher than pre-intervention (P<0.01). After intervention for 12 h, the value was much higher than intervention for 6 h (P<0.05) and pre-intervention (P<0.01). After intervention for 24 h, the value had no statistical difference with the intervention for 12 h (Figure 2A). Rh123 fluorescence intensity began to decrease after TNF-α intervention for 2 h (P<0.05) and the value decreased further after 6 h which was significantly lower than pre-intervention (P<0.01). The difference had no
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Effect of TNF-α on P-gp expression and function in incipient RA group: For P-gp expression, the difference had no statistical difference between TNF-α intervention for 2 h and pre-intervention. Compared with pre-intervention, the value increased significantly after intervention for 6 h (P<0.05) and 12 h (P<0.01). The value had no statistical difference between intervention for 12 h and 24 h (Figure 3A). Rh123 fluorescence intensity began to decrease after TNF-α intervention for 2 h (P<0.05) and the value decreased further after 6 h which was significantly lower than pre-intervention (P<0.01). The difference of Rh123 fluorescence intensity had no statistical significance between 12 h and 24 h (Figure 3B).

P-gp expression and function in effectively cured RA group: For P-gp expression, the difference had no statistical difference between TNF-α intervention for 2 h and pre-intervention. After intervention for 12 h, P-gp expression was significantly higher than pre-intervention (P<0.01). The value had no statistical difference between intervention for 12 h and 24 h (Figure 4A). Rh123 fluorescence intensity began to decrease after TNF-α intervention for 2 h (P<0.05) and the value decreased further after 6 h which was significantly lower than pre-intervention (P<0.01). The intensity after intervention for 12 h was markedly lower than the 6 h (P<0.05). The difference of Rh123 fluorescence intensity had no statistical significance between 12 h and 24 h (Figure 4B).

P-gp expression and function in refractory RA group: For P-gp expression, the difference had no statistical difference between TNF-α intervention for 2 h and pre-intervention. After 6 h, the value increased much higher compared with pre-intervention (P<0.01). Compared with the 6 h, the difference had no statistical significant with intervention for 12 h and 24 h (Figure 5A). Rh123 fluorescence intensity began to decrease after TNF-α intervention for 2 h (P<0.05). After 6 h, the difference had statistical significant with pre-intervention (P<0.01) and intervention for 2 h (P<0.01). Compared with the 6 h, the difference had no statistical significant after intervention for 12 h and 24 h (Figure 5B).

Comparison of P-gp expression and function between each group: The results above showed P-gp expression reached the highest after TNF-α intervention for 12 h in each group and maintained at the high level. As for Rh123 fluorescence intensity, it reached to low level after TNF-α intervention for 12 h and maintained at the low level further. Therefore, we compared P-gp expression and Rh123 fluorescence intensity between each group after TNF-α intervention for 12 h. Results showed P-gp expression in RA groups was significantly higher than the control (P<0.01). The value in refractory RA
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Discussion

Patients with RA are known to have increased mortality rates compared to the general population, and cardiovascular disease is the most important cause of death [15]. Both traditional and nontraditional risk factors contribute to atherogenesis in RA [16]. The first approved biologic agents for the treatment of RA were inhibitors of tumour necrosis factor (TNF) group was the highest and reached to the lowest in effectively cured RA group (Figure 6A). Rh123 fluorescence intensity in RA groups was significantly lower than the control (P<0.01). In RA groups, the intensity reached to the highest in effectively cured RA group and lowest in refractory RA group (Figure 6B).

Figure 5. Influence of TNF-α on P-gp expression and Rh-123 fluorescence intensity in refractory RA group. A: P-gp expression after TNF-α intervention for 0, 2, 6, 12 and 24 h; B: Rh-123 fluorescence intensity after TNF-α intervention for 0, 2, 6, 12 and 24 h; “P<0.05, compared with 0 h, the difference has statistical significance; “P<0.01, compared with 0 h, the difference has significant statistical significance; “P<0.05, compared with 2 h, the difference has statistical significance; “P<0.01, compared with 2 h, the difference has significant statistical significance; “P<0.05, compared with 6 h, the difference has significant statistical significance; “P<0.01, compared with 6 h, the difference has significant statistical significance.

Figure 6. Comparison of P-gp expression and Rh-123 fluorescence intensity in the 4 groups after TNF-α intervention for 12 h. A: Comparison of P-gp expression in the 4 groups; B: Comparison of Rh-123 fluorescence intensity in the 4 groups. “P<0.05, compared with pre-intervention group, the value in Intervention 12 h group has significant difference.
duct mucosa of hepatocyte cells and so on. It strongly indicated PgP play an important role in the body excreting toxic compounds dysplasia and metabolic waste to urine, bile and enteric cavity. Recently studies reported PgP participate in regulating molecule metabolites, proliferation, differentiation and other ways, which indicated PgP potentially have multiple physiological functions [20].

In this research, to study the effect of TNF-α on PgP in resistant rheumatoid arthritis (RRA), we divided RA patients into 3 groups: incipient RA, effectively cured RA and refractory RA group. Samples in the control were the normal. First, we determined PgP expression by FCM, PgP function by Rh-123 accumulation assay, mRNA expression of PgP in PBMC by RT-PCR and TNF-α level in serum by ELISA. Results showed PgP expression and function, TNF-α mRNA expression and serum TNF-α level in the control were all much lower than RA groups. In RA groups, the values in refractory RA group were much higher than the other groups, and values in effectively cured RA group were the lowest. Correlation analysis showed there was a positive correlation between PgP expression and mRNA expression of TNF-α, PgP expression and serum TNF-α level, PgP expression and DAS28 score. Rh-123 fluorescence intensity had a negative correlation with mRNA expression of TNF-α, serum TNF-α level and DAS28 score. Those results indicated PgP may participate in the formation of MDR of RRA. TNF-α may play an important role in PgP expression and function. Thus, interference by TNF-α on PBMC from the 4 groups for 2, 6, 12 and 24 h was performed. Pgp expression and function was determined by FCM and Rh-123 accumulation assay, respectively. Results showed the expression and function of Pgp were up-regulated by TNF-α. The expression and function of Pgp in refractory RA group were sensitive to the stimulation of TNF-α with same concentration in RA groups. This indicated TNF-α may mediate the MDR of RA and promote the formation of RRA. Previous studies showed Pgp was associated with RA. Cusson studied on 68 patients with RA and 44 the normal, and found patients with high Pgp expression in peripheral blood lymphocyte to drug therapeutic effect was not good enough compared with those patients with low Pgp expression [21]. Other study showed Pgp activity was relevant with the drug sensitivity of RA to glucocorticoids [22]. Study by Tsujimura S et al. showed drug resistance of RA patients was related with Pgp expression in lymphocyte [23]. In our study, Pgp expression occurred in the normal and RA groups, but the value was much higher in RA groups, which is consistent with the previous studies [24]. So, we speculated Pgp promoted the formation of MDR in RA. TNF-α is a pro-inflammatory cytokines in RA. Its expression increased in synovial membrane and synovia of RA, and the increased degree of TNF-α was associated with the activity and severity of disease, which can be used as judgement index for disease activity [25]. It can interact with a variety of tissue factors and matrix proteins to promote the inflammatory response, abnormal synovial cell proliferation and apoptosis in RA. Many studies showed TNF-α participates in Pgp regulation and the formation of MDR. The regulation of TNF-α on Pgp can not only in transcriptional level, but only in translational and the modification level after translation [26].

Study on chronic inflammatory diseases and animal experiments with central nervous system drugs transferred by Pgp showed TNF-α can up-regulate Pgp expression and activity in mouse brain capillaries [27]. Large sample multicenter clinical trials showed TNF treatment can improve the symptoms of RA, reduce disease activity, and especially in RRA treatment on TNF greatly improved the prognosis of RA [28]. Study by Badger et al. showed the expression and activity of Pgp decreased in patients with RRA after etanercept (TNF receptor fusion protein) treatment, the activity of disease is under control, which suggested anti-TNF-α treatment can decrease Pgp expression and activity, and reverse MDR mediated by Pgp [29]. Thus, we speculated TNF-α may participate in Pgp regulation and the formation of MDR in RA.

In conclusion, Pgp expression and function were associated the formation of MDR in RA. TNF-α can up-regulate PBMC Pgp expression and function in patients with RA, mediate the MDR of RA and participate in the formation of RRA.

Disclosure of conflict of interest

None.

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