

DRD1-DRD5 EXPRESSION PROFILES IN ARTHRITIS RHEUMATOID PATIENTS USING REAL TIME PCR

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Abstract

Objectives

The cause of rheumatoid arthritis (RA) as a chronic inflammatory autoimmune disease is still unknown. It appears that both genetic and environmental factors play a role in its pathogenesis. Recent studies reveal that in addition to the CNS, immune cells synthesis neurotransmitters so that these catecholamines can regulate immune functions. The aim of this study is to evaluate the dopamine receptor gene expression profiles on peripheral blood mononuclear cells of rheumatoid arthritis patients in comparison with normal individuals.

Material & Methods

In the present study, we investigated dopamine receptor gene expression in PBMCs of 40 RA patients and 40 healthy individuals using Real Time-PCR. The specificities of the obtained Real time PCR products for the respective dopamine receptors fragments were confirmed by sequenced analysis capillary system

Results

We found that DRD1-DRD5 types of dopamine receptors genes expression profiles of rheumatoid arthritis patients differ compared to healthy individuals. Moreover, a significant difference of DR2 and DR4 gene expression was seen in rheumatoid arthritis patients.

Conclusion

This study showed that some types of dopamine receptors genes expression profiles alter in rheumatoid arthritis patients with comparison to healthy individuals. Moreover, this alteration possibly could result in dysfunction of dopaminergic system in immune cells and finally lead to rheumatoid arthritis.

Keywords: Rheumatoid arthritis, Dopamine receptor, Gene expression, Human peripheral blood lymphocytes, Real Time- Polymerase Chain Reaction

Introduction

Rheumatoid arthritis is traditionally considered a chronic, inflammatory, multisystem, autoimmune disorder that causes the immune system to attack the joints. It is a disabling and painful inflammatory condition, which can lead to substantial loss of mobility due to pain and joint destruction. RA which affects about 1% of the population in worldwide is two to three times more frequent in women than men and can lead to disability and reduced quality of life (1). The cause of RA is still unknown, but both genetic and environmental factors appear to play a role (2). It is suspected that susceptibility to rheumatoid arthritis is an inherited trait. The association with certain human leukocyte antigen (HLA) and

RA has been recognized for a long time (3), and it is found that rather specific amino acid sequences, the so-called shared epitope (SE) confer the highest risk for developing RA (4). Recently in a study of blood bank donors it was demonstrated that approximately half of patients with RA had specific serologic abnormalities (rheumatoid factor and anti-CCP antibodies) several years before the onset of symptoms (5). The earliest events in RA might involve activation of the innate immune system, which triggers a T-cell response possibly directed towards citrullinated proteins (6). Recent studies reveal that in addition to CNS, immune cells can also synthesis catecholamines and that catecholamines can regulate immune functions.

Human peripheral blood lymphocytes (HPBL) express dopamine receptors and dopamine transporters and synthesize indigenous dopamine through tyrosine hydroxylase dependent pathway. Five different dopamine receptor genes (DRD1, DRD2, DRD3, DRD4, and DRD5) have been recognized and cloned. Whereas DR1-like family receptors (DR1 and DR5) are excitatory, DR2-like family (DR2-DR3, DR4) are inhibitory (7). Changes in the dopamine system are influenced not only by dopamine itself, but also by dopamine receptors that are encoded by five different dopamine receptor genes (DR1-DR5). Although the expression of the dopamine receptors is well characterized in the brain but little work has been done to examine their expression in other organ tissues. Despite the fact that in certain diseases of the nervous systems alterations in dopamine receptors gene expression in different cells have been reported (8,9), but there is not any evidence that reports investigation on dopamine receptors gene expression in peripheral blood lymphocytes in RA patient. The aim of the present study was to investigate dopamine receptor expression in peripheral blood lymphocytes of RA patients against the background of the hypothesis, that a persisting dysfunction of the dopaminergic system contributes a biological cause to the chronic character of autoimmune system diseases such as RA.

Material & Methods

Patients with established RA attending outpatient clinics at the Rheumatology Department, Taleghani Hospital, Tehran, Iran, were enrolled in this study between 2006

and 2008. All participants gave informed consent. All data used for this study were documented at inclusion. Eighty individuals (aged 25-55 years) including forty normal individuals and forty patients (31 males, 9 females) took part in this study. For the controls, age and sex matched healthy persons considered. All patients fulfilled the ACR criteria and any patients with a previous history of neurologic, neuropsychologic, or autoimmune disease excluded. All subjects were Iranian peoples that had minimum disease duration of 1 year, and also at least one definite radiographic erosion on their hands or feet (10).

Peripheral blood samples (6ml) were obtained from the cubital vein and collected in cell preparation tubes containing an anticoagulant (0.05 M EDTA). Peripheral blood mononuclear cells (PBMC) were isolated from 3ml of each blood sample by Ficoll-Hypaque density centrifugation (Lymphoprep™, Oslo, Norway) which its density was 1.077 ± 0.001 g/ml (20°C) and its osmolality 290 ± 15 mOsm. Horizontal swing out centrifuge was used for cells isolation with 2500 rpm, at 20 min and 1. speed regulation. The buffy coat was collected and washed three times in Phosphate buffer saline (PBS). The total mRNA was isolated from PBMC by High pure RNA isolation Kit (Roche, Germany), according to the manufacturer's instructions and the amount and purity of RNA were determined by spectrophotometry (11). For synthesis of cDNA, 11 µg of total RNA extracted from lymphocytes was reverse transcribed into first-strand cDNA by using M-MuLV RT (recombinant moloney murine leukemia virus reverse transcriptase) according to the manufacturers protocols (Revert Aid™ First Strand cDNA Synthesis Kit Fermentas, EU). Samples were reverse transcribed with oligodT primers (12). The cDNA can be stored up to one week at -20°C. For quantitative analysis of extracted RNA were examined by electrophoresis on agarose gel and optical density was quantitated by using densitometric scanning imaging system. For identity confirmation of synthesized cDNA, common PCR reactions were run for 26 cycles using the following conditions: at 94 °C for 10 second (s), at 58 °C for 10 s, at 72 °C for 30 s, and at 72 °C for 5 min. The PCR products were visualized by gel electrophoresis on a 2% agarose gel to confide concentration of cDNA for each sample. Reactions also were carried out in a

Real Time-PCR (Roach, Germany) with a Cyber green fluorogenic nucleotide to monitor cDNA amplification by the increase in Fluorescence intensity and with using primer pairs specific for five dopamine receptors (DRD1-DRD5) mRNAs and β -actin as internal control (All reagents Prepared from Fermentas, EU) (Table1). The reaction condition of Real Time-PCR for each of D1-D5 receptor and β -actin genes was different. The specificities of the obtained Real Time-PCR products for the respective dopamine receptors fragments were confirmed by capillary sequenced analysis (ABI3700, Applied Bio system, USA) (13).

Statistical analysis

We used SPSS 16.0 (SPSS, Inc, Chicago, IL) and test to analysis the data of RA patients in comparison of healthy individuals. Relative mRNA expression calculated with the $\delta\delta$ Ct-method (14).The significant value in this study was less than 0.05 ($P \leq 0.05$).

Results

We examined detection of the mRNA expressions of dopamine receptors in PBMC with highly sensitive methods. We focused on the all subtypes of dopamine receptors (DRD1-DRD5). Expression of the different dopamine receptors genes segments was studied by analyzing mRNA extracted from the samples. In order to detect dopamine gene receptors expression on RNA level, Real time PCR was performed for the regions of different dopamine receptors. At the end, the Real Time-PCR products were sequenced by capillary sequenced analysis. Therefore, results revealed that all dopamine receptors genes subtypes were expressed on PBMC of patients and healthy individuals. The size of dopamine receptors gene-specific amplicon was different on gel electrophoresis. They consist from left to right: lane 1; Molecular Weight Marker dopamine receptor, Lane 2; DRD1 (size 471 bp), Lane 3; DRD2 (size 521 bp), Lane 4; DRD3 (size 670 bp), Lane5; DRD4 (size 153), and

Table1.Primer sequences of Dopamine Receptors & β -actin

Gene	Sequence FP	Sequence RP	Amplicon Size	NCBI Reference Sequence
DRD1	5'AAACCCACAAGCCCCTCTGA 3'	5'GATGAATTAGCCCACCCAAAC 3'	471 bp	NM_000794.3
DRD2	5'GCGGACAGACCCCACTACAA 3'	5'AAGGGCACGTAGAAGGAGA 3'	521 bp	NM_000795.2
DRD3	5'CCGCATTTGCTGTGATGTT 3'	5'TTTGGGTTGCCTTCTTCT 3'	670 bp	NM_000796.3
DRD4	5'CCTGCGGCTCCAACGTGTC 3'	5'GGAAGCCCCGACCACCAC 3'	153 bp	NM_000797.2
DRD5	5'AACCTGTGCGTCATCAGCG 3'	5'CAGATCCATGAGGGGGTTT 3'	1078 bp	NM_000798.3
β Actin	5'TGAAGTGTCACGTGGACATCCG 3'	5'GCTGTCACCTTCACCGTTCCAG 3'	447 bp	NM_001101.2

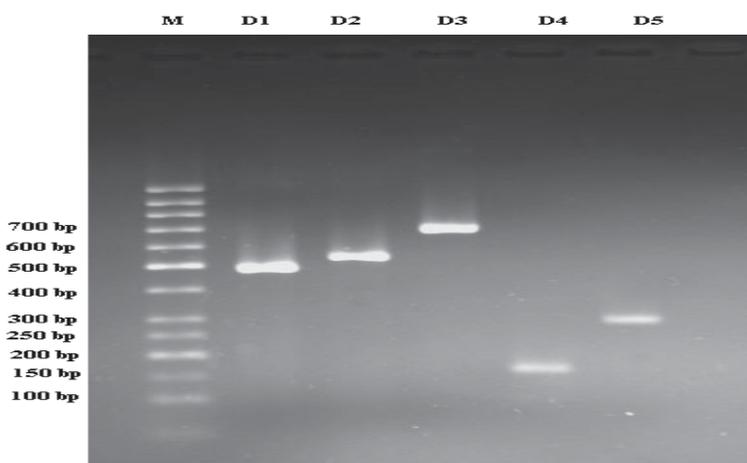


Fig 1: Agarose gel electrophoresis of PCR product of dopamine receptors genes

Lane6; DRD5 (size 1078) (Figure 1). Concentration of samples was calculated. In addition, Mean, Standard deviation and P Value for D1-D5 were determined (Table2).

Table 2. Comparison of the expression pattern of human dopamine receptor genes in RA patient and normal individuals

Dopamine receptor	Normal (Mean ± SD)	RA (Mean ± SD)	P value*
D1	9.1137±14.4261	4.8777±7.0967	0.194
D2	2199.4605±3130.8097	207.4748±820.6691	0.011
D3	109443.28 ±158934.836	1149816.31±1149816.31	0.118
D4	1.0563 ±2.6446	5.2109 ±11.1688	0.014
D5	23129.712 ±27109.3537	20277.913 ±27576.9155	0.674

*All value of dopamine receptor genes concentration is relative to beta actin concentration

However, when RA patients and matched normal individuals were analyzed for D1, D2, D3, D4 and D5 receptors genes expression by Real time PCR and normalized to the housekeeping gene β-actin for each sample with the δδCt method, for RA patients, considering $P \leq 0.05$, we determined a lower expression of D2 receptor compared to the normal individuals group (Pvalue=0.011, Mean±SD+2199.4605±3130.8097 for normal and 207.4748 ±820.6691 for RA patients) (Figure 2); however our analysis showed that PBMC from RA patients express significantly higher amounts of D4 receptor mRNA compared to normal individuals (significant P=0.014, 1.0563±2.6446 for normal and 5.2109 ± 11.1688 for RA patients) (Figure3). No significant differences were found for D1, D3 and D5 mRNA expression between the healthy and patient groups.

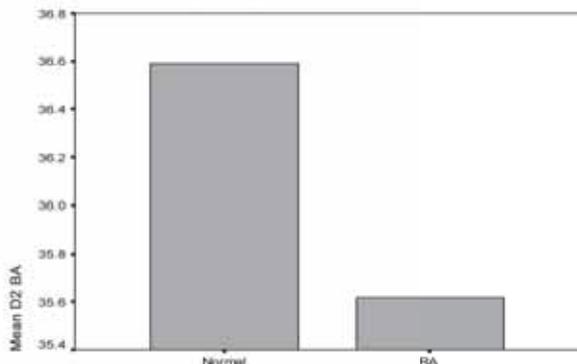


Fig 2: The D2 dopamine receptor gene expression reduced in RA patients.

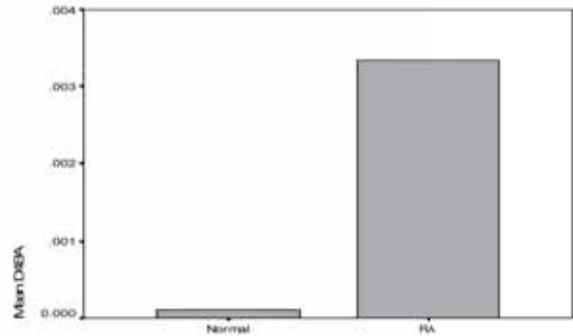


Fig 3: The D4 dopamine receptor gene expression increased in RA patients

Discussion

The bidirectional interactions between the neuroendocrine and immune systems are mediated by anatomical connections and mediators released and recognized by both systems, implying neuroendocrine hormones and neuropeptides produced by immune cells and cytokines by neuroendocrine cells; common receptors expressed on cells of the immune and neuroendocrine systems; effects of neuroendocrine mediators on immune functions; and effects of cytokines on the neuroendocrine system. Autoimmune diseases in general are complex genetic diseases where genes and environment interact in unknown ways (15). Rheumatoid arthritis (RA) is one of the common autoimmune disorders usually considered as a chronic, inflammatory; multisystem that causes the immune system to attack the joints. The cause of rheumatoid arthritis is a very active area of worldwide research (16). Neurotransmitters are signal substances that have traditionally been regarded as mediators of signal states between cells in the nervous system. Whereas the mechanisms of neurotransmitter regulation in CNS are well understood, only recently new evidence come to light elucidating the modulatory role of neurotransmitters in immune function (17). In certain diseases of the immune and nervous systems, alterations in dopamine receptors gene expression in different cells have been reported (18).

Individual vulnerability to develop autoimmune disease is associated with both genetic and environmental factors. Association studies in such patients have explored the contribution of gene variants in the dopaminergic system in these disorders. This system is involved in motor control, endocrinological function, the reward system

and cognition. The diverse physiological functions of dopamine are mediated by five different dopamine receptors. Dopamine receptors belong to a large super-gene family of receptors which are linked to their signal transduction pathways through heterotrimeric G proteins (19). A variety of signaling events are known to be regulated by dopamine receptors including adenylate cyclase and phospholipase activities and various ion channels (20). Disturbances in dopaminergic transmission may cause some disorders, and the receptors are primary targets for drugs used to treat the disorders. The human genome is known to contain five genes encoding the functional dopamine receptors, DRD1, DRD2, DRD3, DRD4 and DRD5, and two genes highly homologous to the DRDS encoding the pseudogenes. Receptors are belonging to two subfamilies; D1 like and D2 like subfamilies. The D1 subfamily consists of two receptors-the D1 and D5, whereas the D2, D3 and D4 receptors comprise the D2 subfamily (21).

Dopamine and its receptor might play a role in the regulation of the immune system. In one study, reverse transcriptase-polymerase chain reaction (RT-PCR) was used to investigate the expression of mRNA for the different subtypes of dopamine receptors in the lymphocytes in the way that D1, D2, D3, D4 and D5 receptor mRNAs were identified. These results provide further evidence for the interaction of dopamine systems and the immune system, and suggest further investigation regarding the immunosuppressive actions of dopamine and dopaminergic drugs might depend on a direct interaction with dopamine receptors on the lymphocyte membrane (22). In previous studies the expression of dopamine receptors on human immune cells has been investigated by using dopaminergic legends, RT-PCR or flow cytometry (23).

It has been repeatedly suggested that dopamine receptor expression in peripheral blood lymphocytes reflects, to some extent, brain status (24). The PBMC could assume as one of subsets of dopaminergic model in neuroimmune system. The aim of the present study was to investigate dopamine receptor expression in peripheral blood lymphocytes of RA patients against the background of the hypothesis, that a persisting dysfunction of the dopaminergic system contributes a biological cause to the chronic character of autoimmune system diseases

such as RA. Also we suggest further investigation on basis of protein detection on separated leukocyte subpopulation which will support this hypothesis more. It is now largely established that the immune and neuroendocrine systems have cross-talk by using their ligands and receptors. Since there is evidence regarding the possible immunological role of these receptors in leukocytes function (25). These results reveal that the molecular dopamine receptors on peripheral lymphocytes are reactive, and that altered expression of dopamine receptor in peripheral lymphocyte of rheumatoid arthritis can contribute to clinically significant diagnosis and treatment of rheumatoid arthritis

In conclusion, our results also demonstrate that dopamine receptors genes are expressed by PBL, particularly in RA, where it localizes on mononuclear cells. The present study showed that peripheral leukocytes in human RA patient group express dopamine receptors including: DRD1, DRD2, DRD3, DRD4 and DRD5 with significant difference in comparison with normal individuals. Our study indicates that although there are no significant differences in the measures of D1, D3 and D5 receptor gene expression of two groups ($P > 0.05$); there are significant differences in D2 and D4 receptor genes between the patient and the control groups ($P < 0.01$). These observed differences may be helpful and can be used for primary diagnosis and treatment of RA.

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