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Expression Profiles of Immune-related Genes in Fluoxetine-treated Human Mononuclear Cells by cDNA Microarray

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To investigate the effect of fluoxetine, one of selective serotonin reuptake inhibitors (SSRIs), on the immune system, human peripheral blood mononuclear cells (PBMC) were treated with fluoxetine (10^{-7} M) for 24 h, and immune-related genes were analyzed by cDNA microarray. Expression of the immune-related genes such as CD107b (LAMP-2), CD47 receptor (thrombospondin receptor), CD5 antigen-like (scavenger receptor cysteine rich family), copine III (CPNE3), interleukin (IL)-18 (interferon-gamma-inducing factor), integrin alpha 4 (CD49d), integrin alpha L subunit (CD11a), IL-3 receptor alpha subunit, L apoferritin, and small inducible cytokine subfamily A (Cys-Cys) member 13 (SCYA13) was induced by fluoxetine. This result suggests that fluoxetine may affect the immune system, and provides fundamental data for the involvement of SSRIs on immunoregulation.

Key Words: Fluoxetine, Immune system, cDNA microarray, Peripheral blood mononuclear cell (PBMC)

INTRODUCTION

Depression can be considered as a psychoneuroimmunological disorder, in which an increase of pro-inflammatory cytokines may have adverse consequences on functional activities of the neurochemical and neuroendocrine systems implicated in the symptoms of the disorder (Irwin, 2002). And, the therapeutic effects of antidepressants are also partly exerted by attenuating the actions of pro-inflammatory cytokines (Castanon et al, 2002). At the clinical level, different classes of antidepressants [e.g., monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs), and selective serotonin reuptake inhibitors (SSRIs)] change the immune system that are associated with depression (Maes, 2001). The antidepressant treatment usually normalizes the changes in both cellular and humoral immunity that occur in depression (Neveu & Castanon, 1999).

The TCA-induced reduction of the production of pro-inflammatory cytokines [particularly tumor necrosis factor-α, interleukin (IL)-1β, and interferon-γ] is usually associated with an increase in the production of the anti-inflammatory cytokine IL-10 (Shen et al, 1999; Connor et al, 2000; Kubera et al, 2000a). An increase in IL-10 release also occurs following chronic SSRIs administration (Kubera et al, 2000b). A number of findings indicates that cytokines are involved in some aspects of antidepressants effect, however, the mechanisms underlying the effects of antidepressants on the immune system are still presently unknown (Beaurepaire, 2002).

Previously, cDNA microarray-based gene expression analysis has been successfully employed to explore the action mechanism and to validate the target genes of several drugs. In the present study, we investigated the effects of SSRI, fluoxetine, on the immune-related genes in human peripheral blood cells using cDNA microarray analysis.

METHODS

Cell and RNA preparation

Fresh heparinized blood sample was taken out from volunteers with a written consent. Equal volume of phosphate-buffered saline (PBS) solution was added to the sample, and 3 ml of Ficoll-Hypaque solution (Sigma, St. Louis, MO) per 10 ml blood/PBS mixture was layered at the bottom of the sample tube. After centrifuging, the mononuclear cell layer was transferred to fresh tube, and mononuclear cells were washed several times by PBS solution. Finally, the peripheral blood mononuclear cells (PBMC) were maintained in RPMI 1640 (Gibco, Grand Island, NY) containing 10% fetal bovine serum in a humidified CO_2 incubator at 37°C.

Fluoxetine hydrochloride (Tocoris, Bristol, UK) at final 10^{-7} M concentration was added to the culture of PBMC, and the cultures were maintained for 24 h at 37°C in a humidified CO_2 incubator.

The total RNA was extracted using RNAzol B kit (Tel-

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ABBREVIATIONS: CD, cluster of differentiation; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; IL, interleukin; MAOI, monoamine oxidase inhibitor; PBMC, peripheral blood mononuclear cells; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressant.

Test Inc., Friendswood, TX). The quality and quantity were measured by absorbance of 260 and 280 nm.

cDNA microarray analysis

Fluorescent cDNA probes were prepared from 10 μg of each total RNA samples using 3DNA array 50 detection kit (Genisphere, Hatfield, PA). For dual channel expression analysis, total RNA sample of control group was labeled by Cy3 dyes and total RNA sample of fluoxetine-treated group was labeled by Cy5 dyes. Fluorescent probes were hybridized to the microarray slide-Human Immunologic chip™ (Creagene, Deajun, Korea) (Ahn et al, 2002). After overnight hybridization at 45°C, non-specific binding probes were washed and the slides were scanned by a ScanArray 5000 scanner (Gsi Lumnonics, Ottawa, Japan). The Cy3 and Cy5 fluorescent intensities of each spots were analyzed by GenePix Pro software (Axon Instruments, Foster city, CA). For the normalization of data, we averaged intensities of all spots obtained with Cy3 and Cy5 in each of the DNA spot (10 internal control genes) and adjusted intensity of each corrected DNA spot as the average intensity ratio Cy3/Cy5=1.0. The criteria for inclusion of cDNA in a group as either induced or repressed expression were based on whether the balanced differential expression was greater than 2 in either direction. For the significance of statistics, the labeling dyes were swapped and hybridization was repeated as above.

Reverse transcription-polymerase chain reaction (RT-PCR)

Thirty microliters of the reaction mixture containing 1 μg of total RNA, 2.5 μM oligo (dT), 1 mM dNTP, 20 U of RNasin, 100 unit of AMV reverse transcriptase (Promega, Madison, WI) and RT reaction buffer (50 mM Tris-HCl at pH 8.3, 50 mM KCl and 5 mM $MgCl_2$) were incubated at 42°C for 1 h, then heated to 95°C for 5 min to denaturate RNA-cDNA hybrids. All samples were reverse-transcribed by the same set of experiments, and the efficiency of the reaction was modulated by glyceraldehydes-3-phosphate dehydrogenase (GAPDH) amplification.

The PCR amplification was performed with hot start. The primer pairs and the predicted sizes of the amplified PCR products are shown in Table 2. Temperatures and time schedules were: 5 min at 95°C for initial denaturation, 30 (for CD47 receptor) or 35 (for IL-18) or 25 (for GAPDH) cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec. The PCR amplification was performed in the Perkin-Elmer GeneAmp PCR system 2400. The amplified products were electrophoresed on 2% agarose gel, and visualized with ethidium bromide for fragment size estimation. The final amount of RT-PCR product was densitometrically calculated using Molecular Analyst™ software version 1.4.1 (BioRad, Hercules, CA).

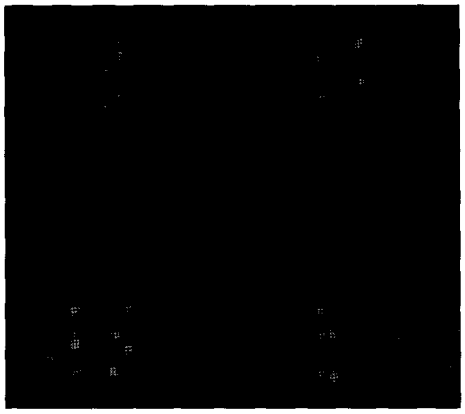

Fig. 1. Two-color image of cDNA microarray. The array consists of PCR-amplified cDNA clones were printed on glass; the diameter of each spot is 500 μm. A sample mixture consisting of Cy3-labelled mRNA from untreated cells and Cy5-labelled mRNA from fluoxetine-treated cells was added. After scanning for each fluorescent dye, false color images (red for Cy3, green for Cy5) were superimposed. Yellow dots represent genes that show no change in expression. The hybridized microarray was scanned with the confocal laser scanner Scan Array 5000 (General Scanning) at 543 nm (Cy3, GHeNe laser) and 632 nm (Cy5, RHeNe laser). Two independent experiments were performed.

RESULTS

cDNA microarray analysis

In order to assess the expression profiles in the PBMC treated by fluoxetine, we used cDNA microarray (Human Immunologic chip™) that contains duplicated cDNA probes from 374 immune-related genes and 10 control genes. Fig. 1 showed a part of the image of Human Immunologic chip™. A difference of more than 2-fold in the normalized intensity ratio was considered significant, and only consistent results after dye-swap experiment were selected (Table 1). Fluoxetine treatment resulted in changes of gene expression in ten out of total 374 immune-related genes: The mRNAs of CD107b (LAMP-2), CD47 receptor (thrombospondin receptor), CD5 antigen-like (scavenger receptor cysteine rich family), copine III (CPNE3), IL-18 (interferon-gamma-inducing factor), integrin alpha 4 (CD49d), integrin alpha L subunit (CD11a), IL-3 receptor alpha subunit, L apoferritin, and small inducible cytokine subfamily A (Cys-Cys) member 13 (SCYA13) were up-regulated compared to control. The L apoferritin gene was shown to have the highest normalized intensity ratio (4.26±0.09) (Table 1). There was no gene down-regulated below 2-fold expression

Table 1. List of gene expression up-regulated by fluoxetine among the 374 immunologically related gene subsets on microarray

Name	Genbank accession no.
CD107b (LAMP-2)	NM_013995
CD47 receptor (thrombospondin receptor)	NM_001777
CD5 antigen-like (scavenger receptor cysteine rich family)	NM_005894
Copine III (CPNE3)	XM 005196
IL-18 (interferon-gamma-inducing factor)	D 49950
Integrin alpha 4 (CD49d)	BC 016671
Integrin alpha L subunit (CD11a)	NM_002209
IL-3 receptor alpha subunit	NM_002183
L apoferritin	X 03743
Small inducible cytokine subfamily A (Cys-Cys) member 13 (SCYA13)	XM 00841

Table 2. Primer pairs used in RT-PCR

Gene	Pairs of primer	Product size (bp)
CD47 receptor	F : 5'-ggtgatagcctatatcctcg-3' R : 5'-tcattcatcattccttttga-3'	231
IL-18	F : 5'-ggaaatgaatcctcctgata-3' R : 5'-tcgttttgaacagtgaacat-3'	214
GAPDH	F : 5'-tggtatcgtggaaggactcatgac-3' R : 5'-atgccagtgagttcccgttcagc-3'	450

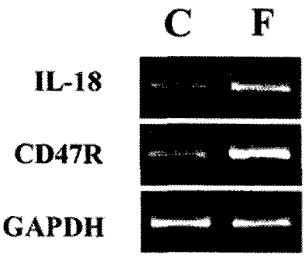

Fig. 2. Confirmation of microarray results by RT-PCR. Two candidate genes, CD47 receptor (CD47R) and interleukin-18 (IL-18), were analyzed by RT-PCR with total RNA from normal (C) and 10^{-7} M fluoxetine-treated PBMC (F). As an internal control, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was amplified.

in this experiment.

Confirmation of microarray findings by RT-PCR analysis

To confirm the high expression of the above 10 genes in fluoxetine-treated PBMC, we performed RT-PCR analysis on two of the 10 genes; CD47 receptor and IL-18. The primer pairs are listed in Table 2. RT-PCR analysis reproduced the results of cDNA microarray. The expression level of CD47 receptor and IL-18 in the fluoxetine-treated PBMC was increased about 2 and 1.5 times, respectively, compared to that in control (Fig. 2).

DISCUSSION

There is some evidence that major depression is accompanied by activation of the inflammatory-response system (Yirmiya, 2000; Dantzer, 2001; Leonard, 2001). It has been hypothesized that increased production of pro-inflammatory cytokines may play a role in the etiology of major depression (Anisman & Merali, 2002; Capuron et al, 2002). Maes (2001) suggested that increased production of pro-inflammatory cytokines is involved in the etiology of depression, and that antidepressive treatments have the immunomodulatory effects.

In the present study, we found that fluoxetine induced expression of several genes encoding immune-related proteins in PBMC. The expression of CD107b, CD47 receptor, CD5 antigen-like, copine III, IL-18, CD49d, CD11a, IL-3 receptor alpha subunit, L apoferritin, and SCYA13 were up-regulated (Table 1). Furthermore, the expression levels of CD47 receptor and IL-18 gene were confirmed by RT-PCR analysis (Fig. 2).

Of the above genes, CD47 receptor is the receptor for adhesion molecule and functions as a self marker on murine red blood cells (Barazi et al, 2002). Oldenborg et al (2000) reported that macrophages might use a number of non-specific activating receptors and rely on the presence or absence of CD47 to distinguish itself from foreign. IL-18 is a pro-inflammatory cytokine that plays a major role in IL-12-driven Th1 cell differentiation (Maiti et al, 2003). IL-3 is a hematopoietic colony-stimulating factor that is capable of supporting the proliferation of a broad range of hematopoietic cell types and also has neurotrophic activity (Hawwari et al, 2002).

Although gene expression profiling using cDNA microarray techniques seems to be an elegant way to identify novel genes or to screen a number of genes simultaneously, some pitfalls and/or study limitations should be considered. There is a potential risk of obtaining false-positive results using this technology.

In conclusion, the results of the present study cannot fully explain the effects of fluoxetine on the immune system, however, we identified differentially expressed immune-related genes in fluoxetine-treated PBMC using cDNA microarray technology. Our results also provide fundamental data for the involvement of antidepressant on immunoregulation. Nevertheless, further in-depth studies are required for understanding the relation between these immune-related genes and fluoxetine.

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