The mRNA Expression Differences of RNA Editing Enzymes in Differentiated and Undifferentiated NG108-15 Cells

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Abstract. The alteration of the editing pattern of serotonin 2C receptor (5-HT2CR) mRNA has been reported during cell differentiation. Since editing of 5-HT2CR mRNA is regulated by adenosine deaminases acting on RNA (ADARs), it is of interest to investigate if the expression of these enzymes changes during cell differentiation. The level of ADAR1 mRNA in NG108-15 cells was decreased by cell differentiation. These results suggest that the decrease of ADAR1 expression during cell differentiation may play an important role in the differentiation-induced alteration of the 5-HT2CR editing pattern in NG108-15.

Keywords: serotonin, adenosine deaminase, RNA editing

The 5-HT2CR (serotonin 2C receptor) is reportedly involved not only in the etiology of neuropsychiatric disorders (1) but also in the cell growth/differentiation (2). Two types of 5-HT2CR mRNA, at least, have been found in the central nervous system; receptor-type (R-) and short variant-type (S-) 5-HT2CR mRNA. The S-5-HT2CR mRNA lacks a sequence that consists of 95-nt and encodes the region from the second intracellular loop site to the fourth transmembrane site (3). It is likely that because of the 95-nt deletion-induced frameshift, the S-5-HT2CR mRNA is translated into a protein with a structure different from the R-5-HT2CR protein. Moreover, this sequence includes the RNA editing site for adenosine deaminase acting on RNA enzyme (ADAR). However, physiological and pathophysiological functions of the protein translated from this S-5-HT2CR mRNA and the roles of ADAR remain to be clarified. We have previously demonstrated that NG108-15 cells expresses S- and R-5-HT2CR mRNA and that the expression of the R-5-HT2CR mRNA is enhanced by a dibutyryl cAMP and phorbol 12-myristate 13-acetate (TPA) treatment-induced neuronal differentiation in NG108-15 cells (4). Analysis of the sequence of the editing sites revealed that editing of the mRNA in undifferentiated cells frequently occurred at sites A and B. Since the adenosine to inosine (A-to-I) editing is regulated by ADAR, it is of interest to investigate if the ADAR mRNA expression changes during cell differentiation. In this study, the ADAR enzyme transcripts in differentiated and undifferentiated NG108-15 cells were quantified by semi-quantitative RT/PCR. NG108-15 cells were cultured under the previously described conditions (5). Total RNA was isolated from the cells cultured in 5 individual dishes by the method of Chomczynski and Sacchi (6). The reversetranscriptation (RT) was performed at 42°C with Superscript II reverse transcriptase and oligo(dT) primer. To determine the bases in the position of RNA editing of 5-HT2CR mRNA (accession No. M21410), PCR was performed with the following primers; 5'-TGGCAGTAAG CATG GAGAAG-3' (forward) and 5'-CGAATTGAAC CGGC TATGCT-3' (backward). Thermocycling was performed at Tm 57°C for 30 cycles. The estimated product size was 275 base pairs. The products were sub-cloned into pBluescript II KS(−) as described previously (5) and was sequenced using a Hitachi-5500 sequencer (Hitachi, Tokyo). Semi-quantitative PCR for ADAR was carried out with the following primer sets and conditions: Primers for ADARI mRNA (accession No. U18121) are 5'-ACT GCT TGA GTG TAGT GAAG-3' (forward) and 5'-GAG AGG AGG AGC ATA GTT CTG AGA CTG-3' (backward). Thermocycling was performed at Tm...
56°C for 20 – 40 cycles. The estimated product size was 615 base pairs. Primers for ADAR2 mRNA (accession No. U7642l) are 5'-CTC TGA GGC CCA CCT GGC C-3' (forward) and 5'-CCA GGA CCA GGC GTG AGAC-3' (backward). PCR was performed at Tm 65°C for 20 – 40 cycles. The estimated product size was 554 base pairs. Primers for β-actin are 5'-AAC GGT CTC ACG TTA GTG TA-3' (forward) and 5'-GTG ACA GCA TTG CTT CTG TG-3' (backward). Thermocycling was performed at Tm 57°C for 16 – 22 cycles. The estimated product size was 222 base pairs. When studying the cycle-dependency of each factor (Fig. 1), five of 1 µg individual RNA samples of culture were mixed and we used the 1 µg of the mixture to make cDNA with oligo(dT) primer, since the quantitative detection can only be achieved when all samples are on the same gel. The products were separated by 6% polyacrylamide gels electrophoresis and stained with SYBR Gold (Molecular Probes, Inc., Eugene, OR, USA). The bands were visualized under UV light and quantified with a densitometer using Densitograph 4.0 software (ATTO, Tokyo). Significance was assessed by single factor analysis of variance followed by Student-Newman-Keuls post-hoc test.

Microscopic observation revealed that undifferentiated NG108-15 cells are polygonal and have a little neurite outgrowth, while the cells differentiated by diBu-cAMP and TPA-treatment are round and have a marked neurite outgrowth (data not shown). We previously reported that RNA editing at site A and B of 5-HT2CR mRNA had occurred more frequently in the undifferentiated NG108-15 cells than in the differentiated cells: the ratios of the editing at the A site are 100% and 30%, and at the B site, 78% and 0% in undifferentiated and differentiated cells, respectively. Figure 1 shows the results of semi-quantitative analysis of ADARs and β-actin mRNA expression level. The RT/RCR fragments of these mRNAs were amplified in a PCR-cycle-dependent manner. The amplification of the β-actin mRNA fragment showed almost the same magnitude in each PCR cycle step (Fig. 1). On the other hand, the amplification of the ADAR1 mRNA fragment in the undifferentiated NG108-15 cells showed a significantly larger magnitude than that of the ADAR1 mRNA fragment in the differentiated cells during the 26th to 31st cycles of the PCR periods. The amplification of ADAR1 mRNA in the undifferentiated and differentiated cells reached the same level following 29 and 31 PCR cycles, respec-

Fig. 1. Quantitative analysis on adenosine deaminase acting on RNA (ADAR) mRNA expression in neuronally differentiated and undifferentiated NG108-15 cells by RT-RCR methods. The figures show the intensities of each band. The mRNA expression of β-actin as the sample-loading control (A), ADAR1 (B), and ADAR2 (C) in undifferentiated (open circle) and differentiated NG108-15 cells (closed circle).
tively. These data indicate that the amount of ADAR1 mRNA expressed in the undifferentiated cells is approximately 4 times more than that in the differentiated cells. The quantification levels in 5 individual cultures were studied at 29 PCR cycles (Fig. 2). The ADAR1 expression was significantly and strongly decreased in differentiated cells. In contrast, significant but weak enhancement of the magnitude of ADAR2 mRNA was found in differentiated cell groups compared with undifferentiated cells (Fig. 2).

5-HT2CR is the G protein coupling receptor, which shows A-to-I RNA editing. Deaminating editing enzymes attack five closely-spaced adenosine residues of 5-HT2CR mRNA that are located within the sequences encoding the second intracellular domain (7). These editing sites are named A, B, E (or C'), C, and D from 5' to 3' (8). A-to-I RNA editing is mediated by a family of ADARs (9). There are three ADAR genes cloned from vertebrates. ADAR1 is capable of editing the A, B, and C, but not D of 5-HT2CR transcripts as determined by an in vitro co-transfection study and by the 5-HT2CR mRNA expression in ADAR1+/−/− tumor. A co-transfection study in human embryonic kidney (HEK 293) revealed that ADAR2 specifically edits at D sites of 5-HT2CR mRNA (7). It is not yet known whether ADAR3 has any enzymatic activity (10). In this study, we found that 5-HT2CR mRNA was highly A-to-I edited, especially at the editing site A and B, and that the editing was inhibited by drug-induced neuronal differentiation in NG108-15 cells. To our knowledge, this is the first report that the expression level of the ADAR1 mRNA was higher in the undifferentiated cells than that in the neuronal differentiated cells. These results further suggested that RNA editing is involved in neuronal differentiation.

We have previously reported that the expression of the S-5-HT2CR mRNA could be detected as well as the R-5-HT2CR mRNA in NG108-15 cells. Although there is no direct evidence, the relationship between an increase of RNA editing and high expression of the S-5-HT2CR mRNA in undifferentiated cells should be noted. The following known facts may support the idea that RNA editing may be involved in diversity of the 5-HT2CR mRNA variant. In the brains of ADAR2−/− mice, the total loss of ADAR2 indeed changed the kinetics of splicing (11). ADAR2 acts on its own adenosine site of its mRNA. This editing results in the adding 47 nucleotides at coding region, and thereby induces the frame shift, indicating that self editing of ADAR2 mRNA regulates its own alternative splicing (11). It could show the possibility that the mRNA editing on 5-HT2CR by ADAR1 may be also involved in the generation of short variant as we discussed in our previous report (5). The sequence of 5-HT2CR cDNA first cloned by Julius et al. (2) reveals that 4 of the 5 editable positions bases are guanosine instead of adenosine residues. The NIH-3T3 fibroblast cells transfected with this edited type of 5-HT2CR cDNA formed the focus (2). Thus it is possible that the S-5-HT2CR/RNA editing of 5-HT2CR mRNA is involved in the cell proliferation.

Gurevich et al. (12) reported that suicide victims with depressive disorder exhibited an enhanced mRNA editing of 5-HT2CR at site E and an inhibited mRNA editing at site D in the dorsal prefrontal cortex compared with normal individuals. Moreover, they found that fluoxetine, an antidepressant, overcomes these editing changes (12). We previously reported that imipramine inhibits cell growth and enhances the expression of R-5-HT2CR mRNA in NG108-15 cells (13). Since neuronal differentiation enhances the R-5-HT2CR mRNA expression (5) and growth inhibition in NG108-15 cells, imipramine may switch the cells from proliferation to
neuronal differentiation, although imipramine dose not affect the RNA editing in itself (M. Tohda et al., unpublished data). These results of interest suggest that control of the RNA editing and the short variant expression of 5-HT2CR mRNA may contribute to switching of NG108-15 cells between neuronal differentiation and proliferation. This mechanism may be also involved in depressive disorder. However, further investigations focusing on the molecular mechanisms and the significance of the short variant/RNA editing during the cell growth/differentiation are required.

References