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N-ACETYLCYSTEINE ENHANCES NEURONAL DIFFERENTIATION OF P19 EMBRYONIC STEM CELLS VIA AKT AND N-CADHERIN ACTIVATION

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We examined whether N-acetylcysteine (NAC) enhanced embryonic body (EB) formation and neuronal differentiation in terms of EB formation, neuronal marker (microtubule-associated protein 2; MAP-2) expression, and neuron maturation using P19 embryonic stem cells. The size and numbers of EBs were greatly increased, together with the up-regulated N-cadherin expression. Also, MAP-2 expression and neurite outgrowth were much increased with activation of serine/threonine protein kinase (Akt) and blocked by addition of an Akt inhibitor (LY294002). Our results suggested that NAC increased EB formation by up-regulating the N-cadherin expression. Furthermore, NAC-enhanced neuronal differentiation was mediated by activation of Akt.

Efficient generation of neurons from neural precursors *in vitro* is of great interest due to the need for a renewable cell source for experimental transplantation studies [1]. Therefore, developing methods to enhance the efficiency of neuronal differentiation various investigations have been conducted [2, 3].

In our study, P19 embryonic stem cells (ESCs) were used, since these cells are thought to reflect early events of neuronal development *in vivo*, and are widely used as an *in vitro* model system for analyzing the regulatory mechanisms of mammalian neuronal development [4]. As it was shown in many studies, treatment of aggregated P19 cells with retinoic acid (RA) at low-concentration (10 nM) induces embryonic cells to differentiate into endodermal and mesodermal derivatives [5, 6].

N-acetylcysteine (NAC) is involved in various cellular events, including induction of Ras signaling which is an important cellular regulator of proliferation, apoptosis and differentiation [7]. However, the effect of NAC on neuronal differentiation of embryonic stem cells has not been reported.

In the presented study, the effect of NAC on embryonic body (EB) formation and subsequent neuronal differentiation was evaluated. The results suggest that NAC specifically activates embryogenesis and neuronal differentiation via increased expression of N-cadherin and activation of Akt, a serine/threonine protein kinase.

EXPERIMENTAL

P19 cell culture and differentiation. To subclone P19 ESCs, 100 cells were inoculated into a 100-mm culture dish suspension in 100 mL of α-Minimum Essential Medium (α-MEM; "GIBCO", USA) supplemented with 2.2 mg/L sodium bicarbonate ("Sigma-Aldrich", USA), 1% antibiotic-antimycotic (AA; "GIBCO"), 7.5% calf serum ("GIBCO"), 2.5% fetal bovine serum (FBS; "GIBCO"), 100 μM pyruvate ("Sigma-Aldrich"), and 1% tyrosine ("Sigma-Aldrich"). P19 ESCs were maintained as undifferentiated cells in tissue culture dishes (Stage 1) or cultured as EBs from 0 to 3 days in the presence of 1 μM RA alone in 0.1% DMSO ("Sigma-Aldrich"), or 1 μM RA and 100 μM NAC ("Sigma-Aldrich") to induce cellular aggregation (Stage 2). The EBs were maintained in precursor medium consisting of α-MEM, 2 mM glutamine, 2.2 mg/L sodium bicarbonate, 1% AA, and 2% FBS. After generation of EBs for 3 days, aggregates were dissociated in 1% trypsin ("GIBCO") and transferred to tissue culture dishes or multiwell-plates or

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Abbreviations: EB – embryonic body; ESCs – embryonic stem cells; MAP-2 – microtubule-associated protein 2; NAC – N-acetylcysteine; p-Akt – phospho-Akt; p-ERK1/2 – phospho-extracellular signal-regulated kinase 1/2; p-JNK – phospho-c-Jun amino-terminal kinase; RA – retinoic acid.
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poly-L-lysine coated glass coverslips for terminal differentiation (Stage 3). Disassociated P19 cells were grown in the absence of RA and NAC. Differentiating cells were maintained in neuronal differentiation medium consisting of Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (DMEM/F12; "GIBCO"), 2 mM glutamine, 1% AA, and 10% FBS. Terminal differentiation was achieved by allowing plated cells to mature for up to 9 days.

Immunocytochemistry. Cells were fixed and permeabilized for 5 min in methanol/acetone (1 : 1, v/v) at room temperature and treated with 0.1 M PBS-3% H_2O_2 to eliminate any endogenous peroxidase activity. The cells were then blocked for 1 h with PBS containing 5% bovine serum albumin (BSA), and incubated overnight with anti-microtubule-associated protein 2 (MAP-2; "Santa Cruz Biotechnology", USA) diluted to 1 : 200. The cells were then immunochemically processed using standard 3,3'-diaminobenzidine (DAB)/nickel staining procedures. Cells on poly-L-lysine coated glass coverslips were mounted with CC/Mount™ ("Sigma-Aldrich") and photographed using a microscope ("Nikon", Japan).

Measurement of neurite outgrowth. MAP-2+ cells from randomly selected areas of at least six cultures from three independent experiments were photographed. Morphological characteristics were quantified using a phase-contrast microscope equipped with a digital camera system and image analyzer ("Olympus", Japan). The length of the primary neurite was defined as the distance from the soma to the tip of the longest process. The total extent of neurite formation was defined as the combined lengths of all neurites per cell. The number of neurites per cell was the total number of processes longer than two cell diameters in length.

Immunoblotting. After 3 days of the P19 ESCs differentiation, the protein levels of N-cadherin, phospho-Akt (p-Akt), phospho-extracellular signal-regulated kinase 1/2 (p-ERK) and phospho-c-Jun aminoterminal kinase (p-JNK) were determined by Western blotting. Cells were washed with ice-cold PBS and homogenized in RIPA buffer containing 1% TX-100, 20 mM Tris (pH 7.5), 100 mM NaCl, 1 mM Na_3VO_4, 40 mM NaF, 5 mM EGTA, 0.2% SDS, 0.5% sodium deoxycholate and 0.2 mM phenylmethylsulfonylfluoride (PMSF). Protein concentrations were determined by the Bradford method using BSA as a standard, and 50 μg of total protein was separated by SDS-PAGE under reducing conditions. The resolved proteins were transferred to nitrocellulose membranes ("Amersham Pharmacia Biotech", UK) and immunoblotted with anti-p-Akt, anti-p-ERK1/2, anti-p-JNK (Thr183/Tyr185), anti-N-cadherin and anti-β-actin (1 : 1000; all antibodies from "Santa Cruz Biotechnology"). The blots were then incubated with HRP-conjugated secondary antibodies (1 : 20000), and bands were detected using the ECL detection system ("Amersham Pharmacia Biotech").

Cell counting and statistical analysis. Immunoreactive cells were counted in 6—12 randomly chosen uniform areas of each well using an eye piece grid at a final magnification of ×40 or ×200. Three to six culture wells were analyzed in each experiment. Data are expressed as mean ± SEM. Statistical comparisons were made by ANOVA and Tukey's post hoc test ("SPSS", USA).

RESULTS

NAC enhances EB formation

To examine the influence of NAC on EB formation, the number and size of EBs were compared after treatment with either RA alone (1 μM) or RA (1 μM) with NAC (100 μM). Significantly more EBs were formed in (RA+NAC)-treated cultures than in the RA alone ones (fig. 1*b* and 1*a*, respectively). Quantitative analysis of EBs, which ranged from 0.05 μm to 0.2 μm in diameter, revealed that NAC+RA treatment dramatically increased the EB diameter in comparison to RA treatment alone (fig. 1*c*). The number of EBs over 0.1 μm in diameter in the NAC+RA group was much greater than that in the RA one.

As shown in fig. 1*d*, the total number of EBs per 100-mm tissue culture dish generated by the NAC+RA combined treatment was significantly greater (2242 ± ± 113.07 EBs/dish) than in the RA only treated dishes (1683 ± 70.65 EBs/dish).

NAC increases the expression of N-cadherin

Adhesion molecules, including N-cadherin, play an important role in embryogenesis as well as in cell-cell interactions mediating neuronal differentiation [8]. To investigate whether N-cadherin mediated signaling promoted embryogenesis under the NAC+RA treatment, the expression pattern of N-cadherin was evaluated by immunoblot analysis. As shown in fig. 2, increased the expression level of N-cadherin was observed at earlier embryogenesis stages (24 h) in (NAC+RA)-treated cultures compared to RA-treated ones. These results are consistent with a greater number of EBs formed in the presence of NAC+RA, relative to RA alone (fig. 1*d*). Interestingly, the up-regulated expression levels were similar in both conditions at later stages (72 h).

NAC enhances the efficiency of neuronal differentiation

To investigate whether enhanced EB formation by NAC directly influenced the level of neuronal cell differentiation, the differentiation level of P19 ESCs by either RA or NAC+RA were evaluated immunocytochemically with a use of the neuronal marker MAP-2. MAP-2 expression in the (NAC+RA)-treated group (fig. 3*b*) was much higher than in the control RA group
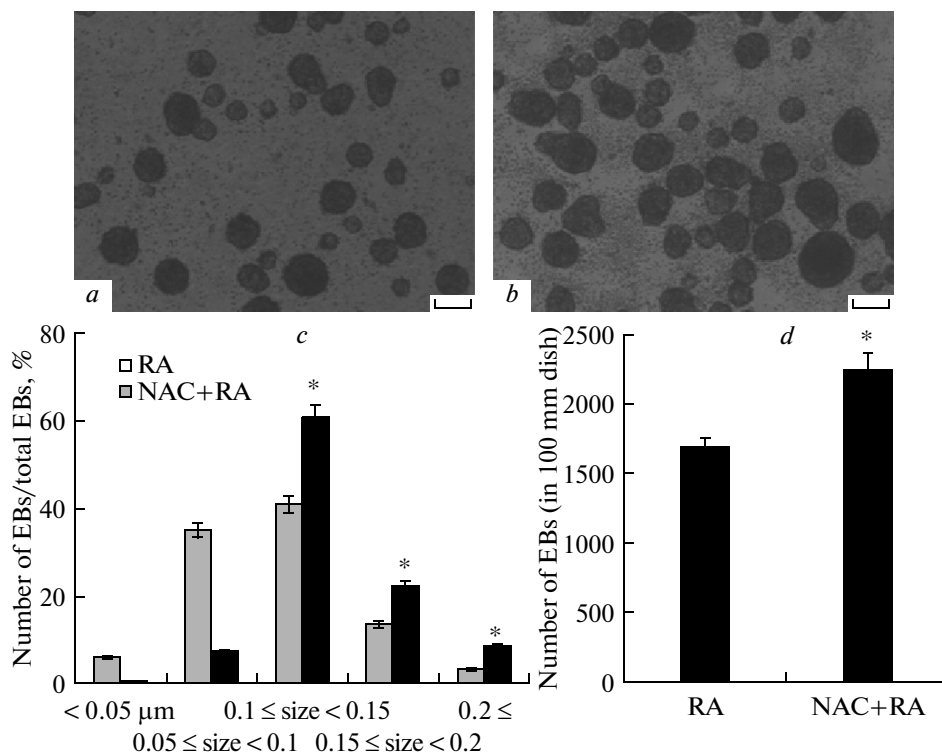
Fig. 1. Effect of NAC on the generation of RA-induced EBs and neuronal differentiation. Representative photomicrographs of EBs generated in the presence of RA (*a*) or RA+NAC (*b*). EBs were classified as clusters of cells with a diameter of 0.05 mm or greater (*c*). The total number of EBs per 100-mm tissue culture dish was scored (*d*). Values are means ± SEM. *Significant differences are $p < 0.05$ versus RA only treatment ($n = 18$). Bar = 0.1 mm.

(fig. 3*a*). In addition, the portion of MAP-2 positive cells was much higher than in the control (fig. 3*c*).

The NAC+RA treatment significantly increased the number of neurites as compared to RA treatment alone (fig. 4*b*). The number of neurites of differentiated P19 cells treated with NAC+RA was twofold greater than of the cells under RA treatment alone. The total neurite length in cells treated with NAC+RA was much greater than in those under RA alone (fig. 4*a*).

### *NAC-induced neuronal differentiation is mediated by Akt*

Immunoblot analysis revealed a significant increase in phospho-Akt in P19 cells aggregated by RA+NAC treatment (fig. 5*a*). Akt kinase activation was sustained over the development of EBs by an additional NAC treatment in both the early (24 h) and late (72 h) stages. On the other hand, the levels of activated MAPK/ERK and JNK were not increased by NAC+RA treatment as compared to those under RA treatment only (fig. 5*a*). Addition of an Akt kinase inhibitor, Y294002 (10 μM) significantly reduced the ratio of MAP-2 positive cells even in the presence of NAC (fig. 5*b*).

DISCUSSION

Results obtained demonstrate that the number and size of EBs were significantly increased by treatment with 100 μM NAC. EB formation is essential and indispensible for embryogenesis and further differentiation [9]. Previous reports have suggested that adhesion molecule mediated cell-cell interactions are involved in establishing and maintaining the neurogenic capability of these cells [10, 11]. In particular, Yagita et al. [12] reported that N-cadherin plays an important role
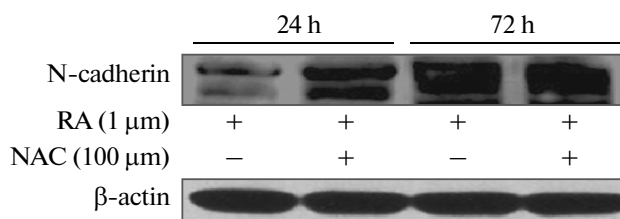
Fig. 2. Adhesion molecules mediate NAC-induced neuronal differentiation of P19 ESCs. Differentiation of P19 cells was induced in the absence (RA) or presence of NAC (RA+NAC), and Western blotting of embryogenesis stage cultures was conducted. NAC+RA treatment increased the expression of N-cadherin in 24-h and 72-h cultures, as compared with the RA only treatment.
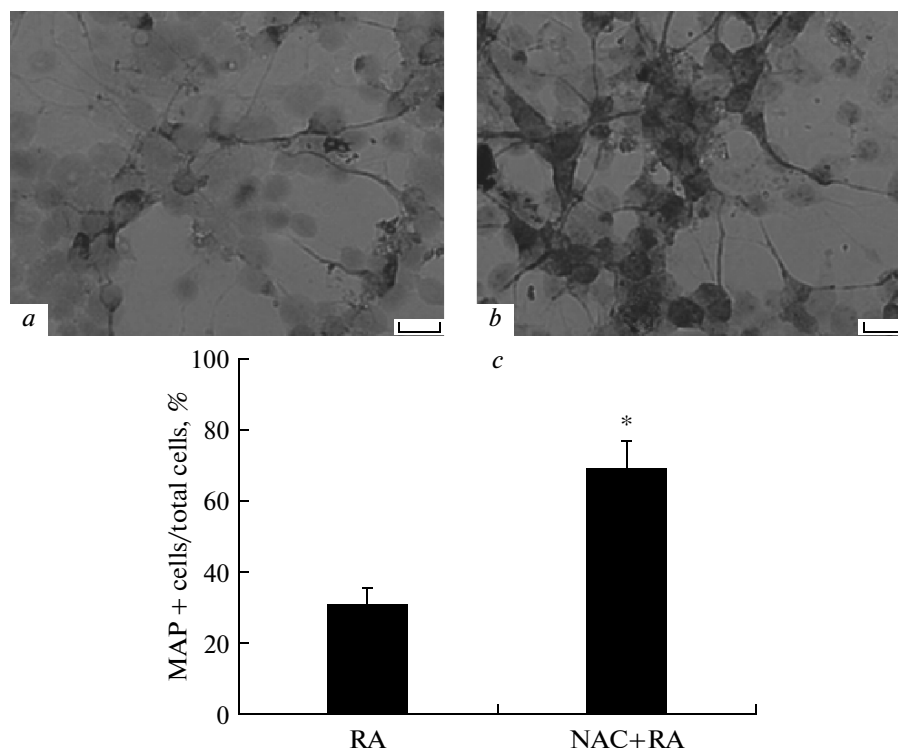
Fig. 3. Characterization of MAP-2-positive cells differentiated from EBs. MAP-2-positive cells were detected in Stage 3 (9-day-old cultures). MAP-2 immunochemical staining of differentiated neuronal cells derived from RA-treated (*a*) or (RA+NAC)-treated (*b*) cultures was performed. The fraction of MAP-2-positive cells was quantified (*c*). Values are means ± SEM. *Significant differences are $p < 0.05$ versus the RA-treated cells. Bar = 100 μm.
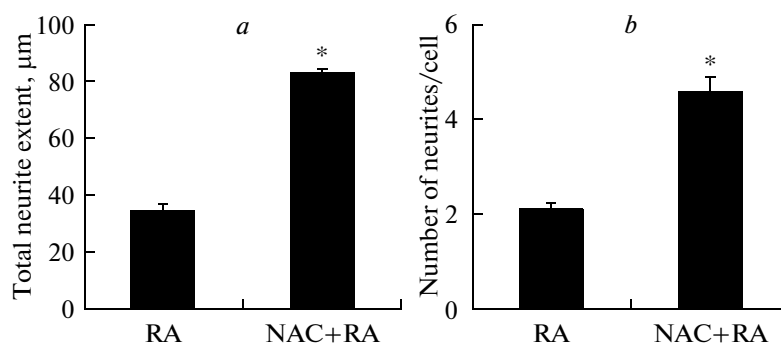

Fig. 4. The effect of NAC on neurite outgrowth in differentiated neuronal cells. Randomly selected fields of labeled neuronal cells were photographed, and neurite outgrowth of differentiated neuronal cells at day 10 was calculated. The total neurite extent is the sum of the lengths of all neurites (*a*), and the number of neurites per cell was counted (*b*). Values are means ± SEM. *Significant differences are $p < 0.05$ versus the RA-treated group ($n = 90$).

in cell cluster formation and cell differentiation regulation. Therefore, we examined whether NAC could enhance EB formation and whether the effect was mediated by N-cadherin. It can be suggested that NAC enhanced EB formation by up-regulating N-cadherin expression at earlier stages as compared to cultures treated with RA only.

To identify the signal transduction pathways that increase the efficiency of (NAC+RA)-induced neuronal differentiation from P19 ESCs, we examined signaling cascades of MAPK/ERK, JNK and Akt

which are involved in various cellular events [13−15]. Levels of Akt activity, as evidenced by phospho-Akt immunoreactivity, were dramatically increased in (NAC+RA)-treated cells compared to RA treatment alone. On the other hand, the levels of activated MAPK/ERK and JNK were not affected by RA+NAC treatment.

Furthermore, treatment with LY294002, an inhibitor of Akt, dramatically reduced the extent of neuronal differentiation induced by NAC. These results
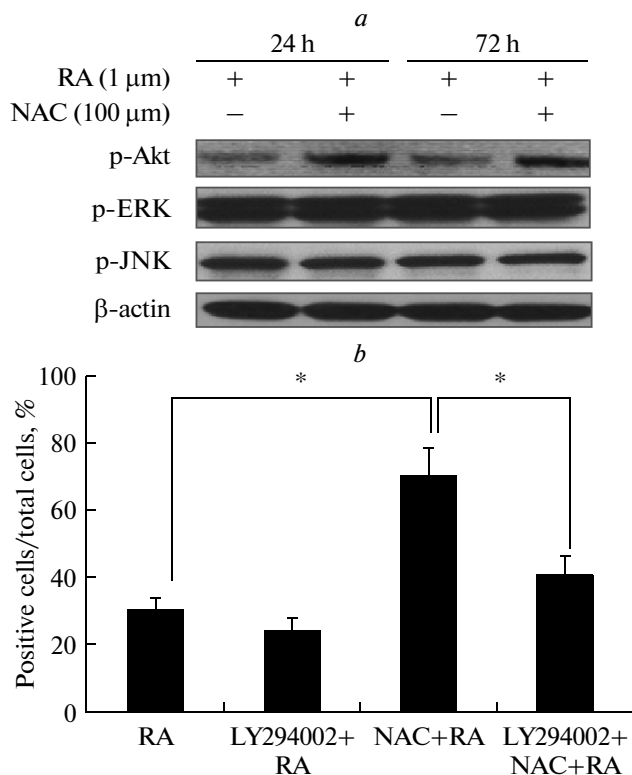
Fig. 5. Akt mediates NAC-induced neuronal differentiation of P19 ESCs. P19 cells were induced to differentiate in the absence (RA) or presence of NAC (RA+NAC). Western blotting was performed using embryogenesis stage cultures, and immunocytochemical staining for MAP-2-positive cells was conducted on Stage 3 (9-day-old cultures). Western blotting revealed that the NAC+RA treatment increased the expression levels of p-Akt in both 24 h and 72 h cultures, but didn't increase neither p-ERK1/2 or p-JNK (*a*) as compared to the RA only. The fraction of MAP-2-positive cells was increased in (NAC+RA)-treated cultures, but decreased in the NAC+RA cultures treated with the Akt kinase inhibitor LY294002 (*b*). Values are means ± SEM. *Significant differences between two groups, RA vs. (NAC+RA) and (NAC+RA) vs. (LY294002+NAC+RA), are $p < 0.05$.

demonstrate that the activation of Akt plays an important role in neuronal differentiation.

NAC has been known as an acetylated precursor of both the amino acid L-cysteine and reduced glutathione (γ-glutamylcysteinyglycine; GSH) [16]. Furthermore, GSH is a major source of reducing equivalents for survival in mammalian cells and tissues [17, 18]. So addition of NAC increased the content of GSH, which may be of a concern with the up-regulation of neurogenesis by addition of NAC.

The effect of NAC as brain medicine has been mostly considered with respect to either anti-inflammation [19], increase of brain-derived neurotrophic factor [20], or anti-oxidative stress [21]. However, in this study, NAC worked as an effective enhancer of neuronal differentiation.

In conclusion, NAC enhanced EB generation through N-cadherin activation and neuronal differentiation via Akt activation during RA-induced P19 ESCs development.

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