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Genistein-Induced Neuronal Differentiation Is Associated With Activation of Extracellular Signal-Regulated Kinases and Upregulation of p21 and N-Cadherin

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Abstract Neuronal differentiation in the mammalian CNS is driven by multiple events. When treated with retinoic acid (RA), hNTera-2 (NT-2) cells undergo postmitotic neuronal differentiation. Here, we show that a prolonged exposure of NT-2 cells with non-cytotoxic doses of genistein, a protein tyrosine kinase (PTK) inhibitor, induced differentiation of NT-2 cells. Additionally, genistein enhanced RA-induced neuronal differentiation by increasing the activation of extracellular signal-related kinase 1/2 (ERK1/2) via phosphorylation at Thr183 and Tyr185 in 3–7 days. Meanwhile, genistein also upregulated N-cadherin and p21 (a Cdk inhibitor), but downregulated proliferating cell nuclear antigen protein (PCNA). MEK1/2 inhibitors, such as PD98059 and U0126, reduced RA-induced ERK1/2 activity, but could not block the genistein effects. Our observations indicate that genistein-induced neuronal differentiation is not dependent of the MEK-ERK signaling cascade. Instead, genistein-upregulated ERK activation is likely due to this chemical's direct effect on chromosome and gene transcription, rather than its inhibition on tyrosine kinases. Failure of inhibition of ERK1/2 activation by the MEK1/2 inhibitors PD98059 and U0126 suggests presence of an unknown activator for ERK1/2 in neuronal cells. J. Cell. Biochem. 96: 1061–1070, 2005.

Key words: genistein; neuronal differentiation; extracellular signal-related kinase (ERK); cell adhesion protein; cyclin-dependent kinase (Cdk) inhibitor p21

Genistein is a potent plant-derived isoflavone that acts like estrogen at low concentrations, but is antiproliferative and antiangiogenic at higher concentrations. The antiproliferative potential of genistein has been utilized in cancer chemotherapy [Kelloff et al., 1996; Polkowski et al., 2000; reviewed by Dixon and Ferreira, 2002]. Genistein is known to induce DNA double-strand breaks, inhibit topoisomerase II [Markovits et al., 1989], and block angiogenesis and protein tyrosine kinase (PTK) activity [Barnes et al., 1994; Fotsis et al., 1995]. Furthermore, genistein modulates differentiation and cell-cycle progression through induction of apoptosis and regulation of PTK activity in many carcinoma cells [Brown et al., 1998; Katdare et al., 2002]. In the nervous system, genistein is demonstrated to protect cortical and hippocampal neurons from oxidative stress or β-amyloid-induced apoptosis [Sonee et al., 2004; Zeng et al., 2004]. Genistein increases the rate of neurite elongation, indicating its neurotrophic potential [Miller et al., 1993; Zhao et al., 2002]. However, genistein-induced neuronal death involves activation of p42/44

Abbreviations used: MAPKs, mitogen-activated protein kinases; ERK1 and ERK2, extracellular signal-regulated protein kinases; PTK, protein tyrosine kinase; NCAM, neural cell adhesion molecule; Cdk, cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen protein; RA, retinoic acid; NT-2, hNTera-2 cells.

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mitogen-activated protein kinases (MAPKs) and calcium accumulation [Linford et al., 2001]. Recent studies also show that genistein activates p38, deactivates extracellular signal-related kinase (ERK), and decreases Cdc25C expression in mammary epithelial cells [Frey and Singletary, 2003]. Whether the MAPK pathway is associated with genistein-regulated neuronal development is largely unknown and remains to be elucidated.

MAPKs are well known to mediate intracellular phosphorylation events linking receptor activation to the control of cell proliferation and stress response, including neuronal development and/or apoptosis [reviewed by Marshall, 1994; Seger and Krebs, 1995; Sano and Kitajima, 1998; Takeda et al., 2000]. Inhibition of MAPKs family of proteins, p42/44 ERK and p38 MAPK activation by interfering MEK1 mutants or inhibitors, blocks neurite formation [Cowley et al., 1994; Kamata et al., 1996; Takeda et al., 2000]. These findings indicate that ERK1/2 or p38 activation is sufficient to induce morphological differentiation. Moreover, cell cycle withdrawal associated with terminal differentiation is regulated by MAPK cascade. Previous studies indicate that ERK1/2 and p38 cooperate to increase expression of $p21^{Cip/WAF1}$, a cyclin-dependent-kinase (Cdk) inhibitor, and inhibition of Cdk [Todd et al., 2004]. p21 blocks the progression of the cell cycle from G1 to S through inhibition of Cdk/cyclin activity and participates in cell differentiation [Casini and Pelicci, 1999]. Proliferating cell nuclear antigen protein (PCNA), a replication and DNA-repair factor, interacts with p21 and Cdk/cyclin to form a quaternary complex [Xiong et al., 1993]. p21 also regulates the PCNA protein level for growth control [Engel et al., 2003]. Because genistein modulates cell-cycle-related kinase functions, here we investigated the effect of genistein on the expression of the cell cycle regulators p21 and PCNA in genistein-induced neuronal differentiation.

On the other hand, generating cell aggregation triggers neuronal differentiation [Honegger and Richelson, 1976; Maar et al., 1997]. Both N-cadherin and neural cell adhesion molecules (NCAMs) are involved in the control of cell interactions and tissue morphogenesis in the nervous system [reviewed by Takeichi, 1995; Edelman and Jones, 1998; Kiss and Muller, 2001]. NCAMs and N-cadherin induce neurite outgrowth or neuronal differentiation, presumably through signaling pathways of Ras-MEK-ERK and fibroblast growth factor receptor [Saffell et al., 1997; Kolkova et al., 2000]. However, the role of genistein on NCAM- and N-cadherin-dependent neurite outgrowth in relative to cell aggregation and MAPK-mediated neuronal differentiation warrants further investigation.

hNTera2/c.D1 (NT-2) cells, a human embryonic carcinoma-derived cell line, exhibits the characteristics of human neuronal progenitor cells after continuous exposure to retinoic acid (RA). It is unclear whether genistein treatment alters RA-induced neuronal differentiation, and whether MAPKs are involved in RA- or genistein-regulated neuronal development. In the present study, we analyzed ERK1/2 and p38 activation, and found that along with ERK phosphorylation, genistein enhanced RA-induced NT-2 neuronal differentiation, upregulated N-cadherin and p21, but downregulated PCNA expression. MEK inhibitors, however, could not block the genistein effect, suggesting that genistein-upregulated ERK activation is likely due to this chemical's direct effect on chromosome and gene transcription, rather than its inhibition on tyrosine kinases.

MATERIALS AND METHODS

Chemicals and Reagents

PTK inhibitors, genistein (4′,5,7-trihydroxyisoflavone), tyrphostin, and daidzein, and tyrosine phosphatase inhibitors, sodium orthovanadate, and phenylarsine oxide, were from Sigma-Aldrich (St. Louis, MO). RA, genistein, and most of the chemicals we used were dissolved in 100% dimethyl sulfoxide (DMSO), then diluted into standard culture medium at 70°C. After they were cooled to 37°C, these solutions were added to cell cultures.

Cell Culture and Drug Administration

Human NT-2 teratocarcinoma cells (Stratagene, La Jolla, CA) were grown at 37°C in a humidified atmosphere of 95% air/5% CO_2, using Dulbecco's modified Eagle medium (DMEM) with nutrient mixture F-12, supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Where indicated, 1.5×10^6 undifferentiated NT-2 cells were grown for 24 h in

10×10 cm^2 Petri dishes, followed by feeding with DMEM/F-12 and 10 μM RA to induce cell differentiation. To investigate genistein-induced neuronal differentiation, RA was replaced by genistein (5–15 μM) in various time-course treatments. Briefly, RA or genistein or other PTK inhibitors are administrated to NT-2 cells in various concentrations 24 h after subculture, and fresh medium and fresh drugs were added daily. For irreversible study, culture medium was replaced by adding genistein-free fresh medium 0.5, 1, 3, 5, 7 days after RA or genistein treatment. ERK1/2 phosphorylation was then analyzed following the drug movement for 0.5–7 days.

To confirm neuronal differentiation, specific antibodies against the following marker proteins were used in immunostaining. They were growth-associated protein 43 (GAP-43), neurofilament medium/light chain (NF-M/L), microtubule-associated protein 2 (MAP2), and neuronal nuclei (NeuN), markers for growth-cone formation or neuroendocrine differentiation (Chemicon International, Inc., Temecula, CA).

Immunoblotting With Phosphotyrosine Antibodies

For measurement of activated or phosphorylated ERK1/2, differentiated cells were lysed in ice-cold lysis buffer composed of 50 mM Tris pH 8.0 containing 150 mM sodium chloride, 1% NP-40, 2 mM PMSF, 2 mM sodium orthovanadate, and 1 tablet of protease inhibitor cocktail (Merck & Co, Inc, Whitehouse Station, NJ) for 25 min at 4°C. Lysates were clarified by centrifugation at 12,000 rpm for 25 min at 4°C. The protein concentration of the cell lysates was determined using a bicinchoninic acid assay KIT (MicroBCA; Pierce Biotechnology, Inc., Rockford, IL) in a microtiter plate. Equal amounts of protein extracts were resolved with SDS–PAGE following the manufacturer's instruction. After electrophoresis, the gel was transblotted onto PVDF membranes (NEN) at 100 V for 1 h at 4°C in a transblot apparatus (Bio-Rad Laboratories, Inc., Hercules, CA). The membrane was blocked overnight in 5% non-fat milk in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) and then incubated with primary antibodies to the indicated protein for 1 h. Antibodies used in the immunostaining were phospho-specific antibody to ERK (Thr-183/Tyr-185) and p38 MAP kinase (Thr-180/Tyr-182) (Sigma; 1:5,000 dilution), anti-N-cadherin, NCAM, and neuronal markers such as MAP2, NeuN, and NF-M (Calbiochem, San Diego, CA). Horseradish-peroxidase-linked-IgG was used as the secondary antibody. Immunoblots were visualized with an enhanced chemiluminescence (ECL) system. Membranes were stripped and reprobed with indicated antibodies to detect proteins. The intensity of protein bands on X-ray films (BioMax, Eastman Kodak, Rochester, NY) were quantified by densitometric scanning and analysis with Image-Quant NT software [Huang et al., 2002; Chen et al., 2004].

For immunofluorescent staining, cells were washed with PBS three times and then fixed with 4% paraformaldehyde in PBS. After washing with NH_4Cl in PBS, cells were permeabilized with 0.5% Triton X-100 for 5 min. Cells were then blocked with 1% BSA in 0.1% Triton X-100, followed by washing three times with PBS, and incubating at 4°C overnight with indicated primary antibodies. For proceeding double labeling, the cells were immersed for 1 h in fluorescein isothiocyanate-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG (Chemicon), and examined under fluorescent microscopy using an appropriate filter [Chen et al., 2005].

RESULTS

NT-2 Cell Differentiation and ERK1/2 Activation Following RA Treatment

Proliferating NT-2 cells in culture exhibited non-process-bearing epithelioid morphology. Compared to control cells, RA suppressed cell growth and altered their morphology. During 1-week culture, the RA-induced NT-2 cells became GAP-43 positive, indicative of differentiating into mature neurons. Similarly, these cells are immunoreactive with other specific neuronal markers NF-M and MAP-2 (data not shown).

To evaluate ERK expression and activation, protein extracts were prepared at various times during RA treatment. ERK1/2 expression between normal and RA groups was similar in a time-course experiment (Fig. 1A). However, ERK1/2 activation (p-ERK) was significantly elevated in the RA-treated group at day 5, indicating that RA causes ERK1/2 phosphorylation (Fig. 1A). Neuronal differentiation was also assessed by NF-M expression. The data showed that RA induced NF-M expression in a

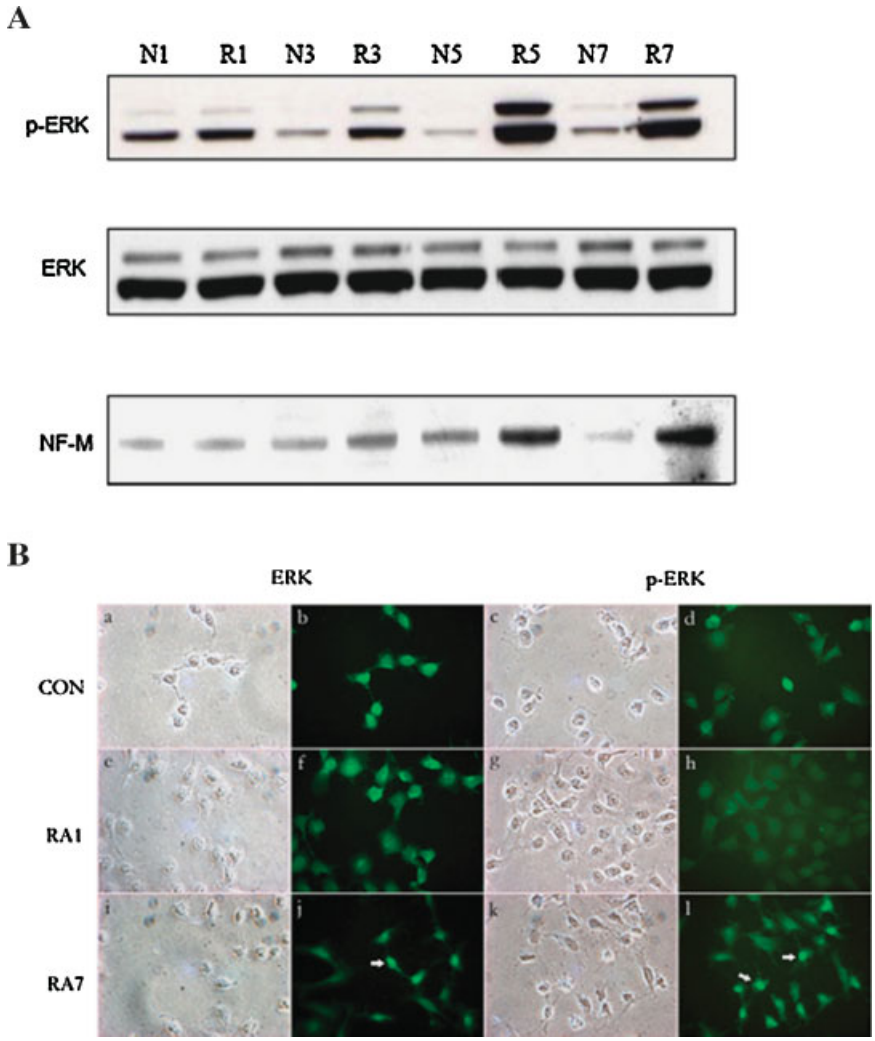

Fig. 1. Comparison of phospho-ERK, ERK1/2, and NF-M protein expression between normal (N) and RA-treated (R) NT-2 cells. In a time-course experiment, there is no significant difference in ERK1/2 expression between normal and RA groups, as determined in Western blotting. However, ERK1/2 activation (or phosphorylation at Thr183 and Tyr185; p-ERK) is significantly elevated in RA group at day 5 in culture (**A**). Immunofluorescent staining shows nuclear localization of p-ERK at day 7 (**B**). Note that the staining intensity in the nuclei (arrows) of RA-treated cells is relatively higher than that of untreated cells.

time-dependent manner. By immunofluorescence, significantly increased nuclear localization of activated ERK (or p-ERK) was shown in RA-treated cells at day 7 in culture, as compared to control and RA-treated cells 1-day post treatment (Fig. 1B). In comparison, activation of p38 and JNK1 was not observed in RA-treated cells (data not shown).

To further investigate the relationship between ERK activation and NT-2 cell differentiation, RA-stimulated NT-2 cells were treated with MEK1/2 inhibitors (PD98059 (PD) and U0126 (U)). These chemicals block phosphorylation of MEK1/2, an upstream kinase for phosphorylating of ERK1/2. After co-incubation with U0126, RA-induced ERK1/2 phosphorylation was downregulated at day 5 and 7 (Fig. 2). Despite the downregulation, the expression of NF-M was not altered, indicating that NT-2 cell differentiation is not altered by U0126. Similarly, PD significantly inhibited ERK1/2 activity (data not shown). However, treatment of the cells with p38 inhibitor SB203580 (SB) had no effect on ERK1/2 activation. Morphologically, SB had no effect on cluster formation of differentiated cells.

Because ERK activation and cell-cluster formation occur during RA treatment, we hypothesize that cell adhesion molecules are involved in cell aggregation. We determined that RA increased N-cadherin expression in a time-dependent manner, significantly upregulated

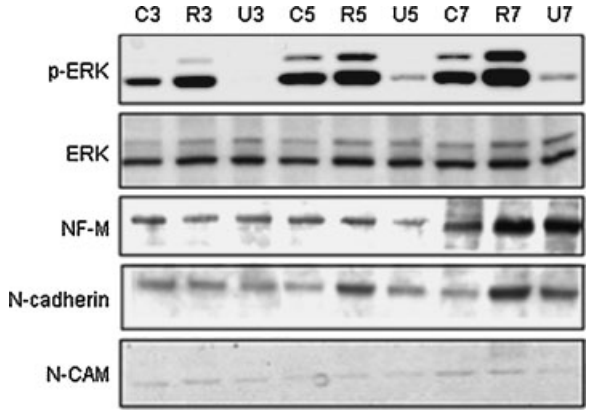

Fig. 2. Immunoblottings showing a downregulation of p-ERK, NF-M, and N-cadherin expression after treatment with MEK inhibitor U0126. No change was observed in NCAM and NF-M expression. C3/C5/C7, R3/R5/R7, and U3/U5/U7 indicate 3–7 days post DMSO (C, control), RA (R), and U0126 (U) treatment, respectively.

from day 5 to 7, and that U0126 suppressed the expression (Fig. 2). However, no change of N-CAM expression was detected after NT-2 cells having been treated with RA and/or MEK inhibitors.

Effect of Tyrosine Kinase Inhibitors on Neurite Outgrowth and Differentiation

Next, we examined the effect of protein tyrosine phosphatase (PTK) inhibitors on NT-2 cell differentiation and neurite elongation, in the presence or absence of RA. At 1 μM, herbimycin was highly toxic to NT-2 cells. At lower concentrations, herbimycin had no effect on NT-2 cell differentiation. Similarly, tyrphostin (10 μM) had no effect. In comparison, PTK inhibitors, such as sodium orthovanadate (10 μM) and phenylarsine oxide (1 nM-1 μM), could not induce neuronal differentiation (data not shown).

As described earlier, cells were routinely subcultured and treated with genistein, also one of PTK inhibitors, which were replaced every day. A prolonged exposure of NT-2 cells to genistein for 10–30 days resulted in induction of NT-2 cell differentiation, as evidenced by the expression of NF-M and NF-L (Fig. 3A). Genistein, in the presence or absence of RA, also induced neurite outgrowth, as evidenced by the presence of GAP-43 (data not shown). Both genistein and RA upregulated N-cadherin

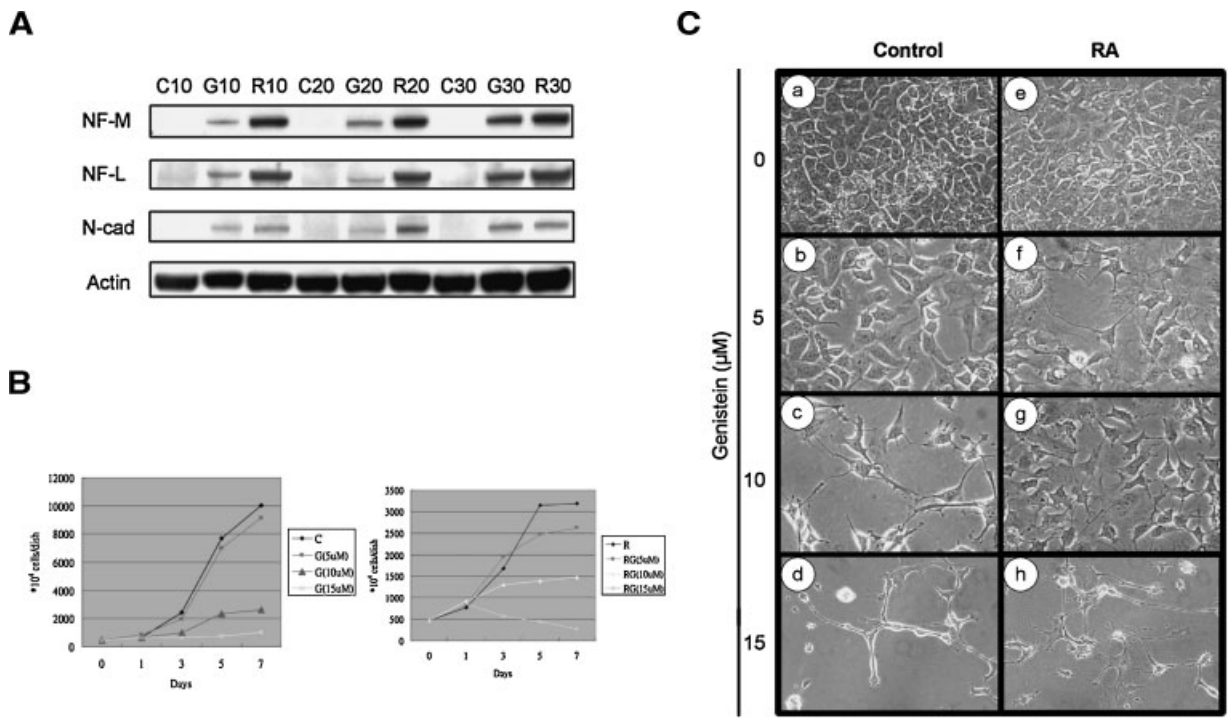

Fig. 3. Genistein-induced neuronal differentiation maintained to the third week, as revealed by upregulation of NF-M and NF-L. An increase in N-cadherin was also detected as shown in RA-induced neuronal differentiation (**A**). High doses (10–15 μM) genistein attenuated neurite outgrowth, whereas NT-2 cell growth was not affected by a low dose (5 μm) (**B**). The rate of cell proliferation shows morphologically (**C**). Compared with controls (**a**, **b** without genistein co-treatment), the length of neurite outgrowth increased with higher dose genistein (5 μm in **c**, **d**; 10 μM in **e**, **f**; 15 μM in **g**, **h**).

protein expression (Fig. 3A), along with cluster formation or cell aggregation (data not shown).

At high concentrations, both RA and genistein are known to inhibit cell growth. Here, we examined the effect of genistein on the rate of NT-2 cell growth. Genistein, at 5–15 μM, inhibited proliferation of NT-2 cells in a dose-dependent manner (Fig. 3B). Similar results were observed with genistein in the presence or absence of RA (Fig. 3B). Genistein induced neurite outgrowth, along with their length increases, in a dose-related manner (Fig. 3C).

Effects of Genistein on the Levels of p21, PCNA, and Cdk5

p21, PCNA, and Cdk5 are markers of the proliferative state of the cells. Here, we examined the potential role of these cell-cycle regulators in genistein-induced neuronal differentiation. p21 expression was shown at day 5 and increased gradually with time in genistein-treated NT-2 cells (Fig. 4A). While genistein upregulated p21 expression significantly, p21 levels were increased slightly in RA-induced NT-2 cells. Notably, reduced expression of PCNA was observed in NT-2 cells after exposure to genistein with time, as compared to control cells (Fig. 4B). The alteration was obvious in genistein-treated NT-2 cells. Further, both RA and genistein upregulated the expression of Cdk5, one of the cyclin-dependent kinases (Cdks) (Fig. 4C). The levels of Cdk5 appear to sustain stably in these treatments with time. The differential expression of cell-cycle-related proteins showed that RA and genistein possibly used various mechanisms for neuronal differentiation.

Genistein Upregulates ERK1/2 Expression and Phosphorylation

Finally, we examined whether genistein induced phosphorylation of ERK1/2 in NT-2 cells. Along genistein-induced neuronal differentiation with time, there was an increased level of phosphorylation or activation of ERK (Fig. 5A). Co-treatment of cells with genistein and RA further increased ERK1/2 phosphorylation (Fig. 5A). We also verified increased phosphorylation of ERK1/2 in dose- and time-dependent manners (Fig. 5B,C). Notably, ERK1/2 activation continued to occur and last for 2–3 weeks in daily genistein-treated NT-2 cells. After genistein removal from the cultured medium for 0.5 to 7 days, upregulation of p-ERK was not attenuated (Fig. 5D). In contrast, RA-treated cells could not sustain ERK1/2 activation effectively after RA removal, indicating that genistein-induced NT-2 cell differentiation is an irreversible event. PD98059 and U0126 downregulated ERK1/2 phosphorylation in control cells (Fig. 5E). However, they were unable to block neurite outgrowth and ERK1/2 phosphorylation in genistein-treated cells (Fig. 5E). These data convincingly demonstrated that the mechanism underlying genistein-activated ERK1/2 is not identical to that of RA.

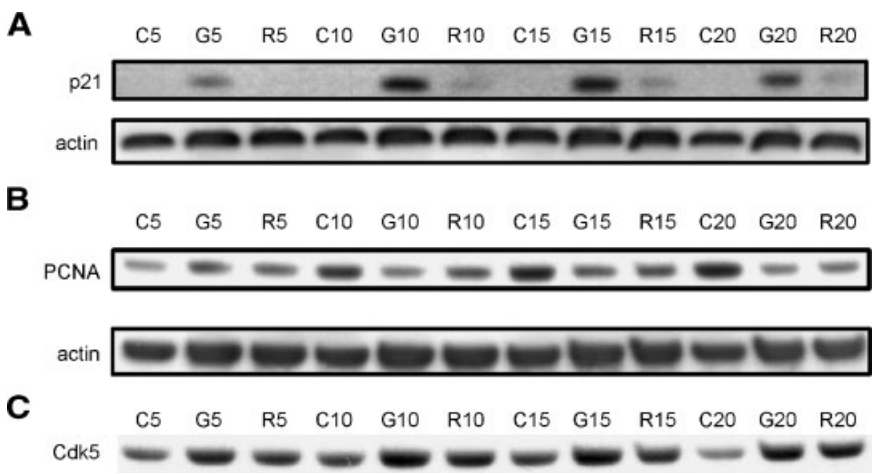

Fig. 4. Genistein increased p21 expression 5–20 days after administration (**A**). Instead, PCNA expression was downregulated in genistein-induced neuronal differentiation (**B**). The effect on p21 and PCNA levels was significant in genistein-treated groups. Cdk 5 expression was upregulated after genistein or RA treatment (**C**). C, control; G, genistein; R, RA.

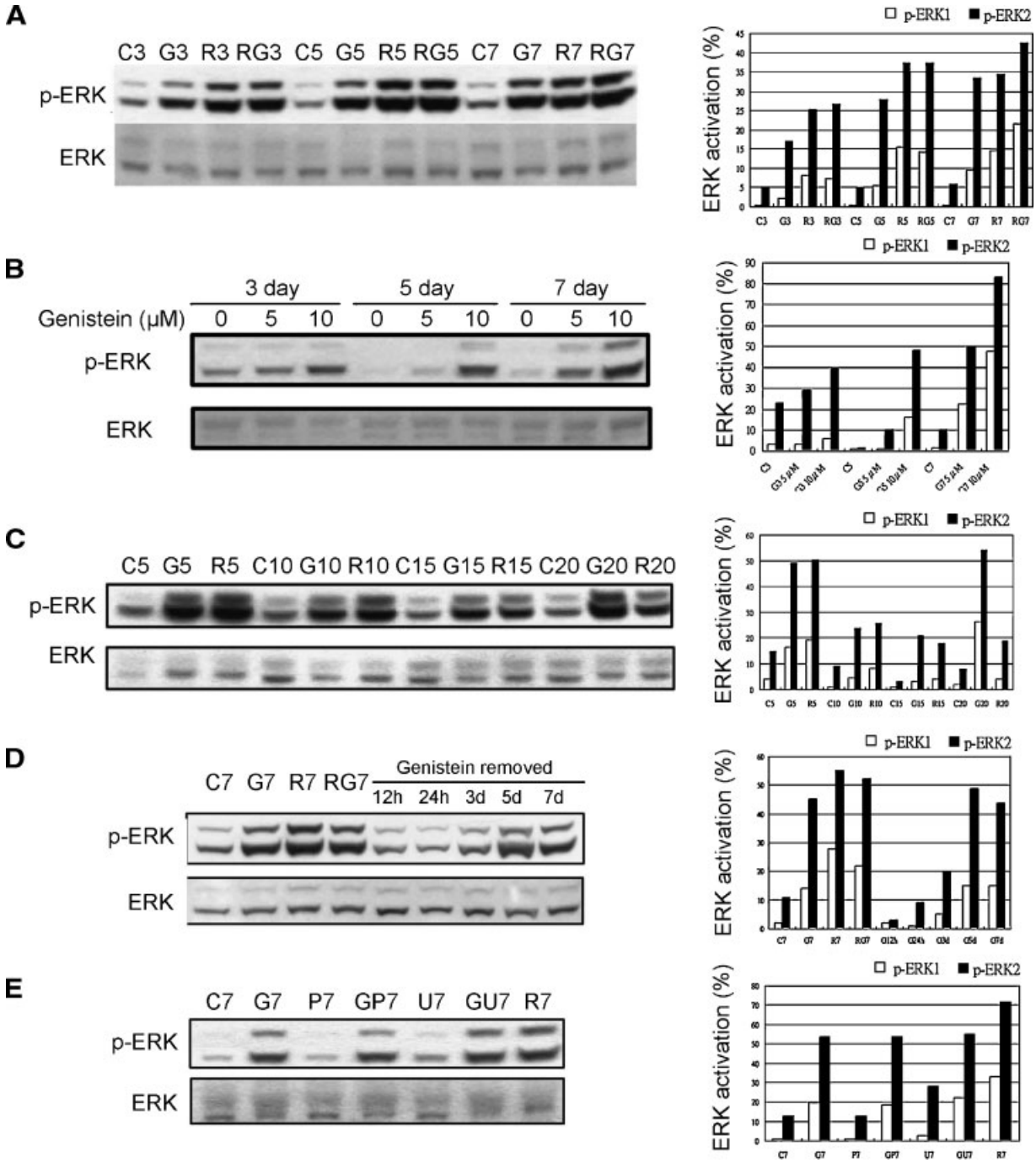

Fig. 5. Genistein increased ERK1/2 phosphorylation in NT-2 cells 2 to 3-week post-culutre. With or without RA induction, genistein administration upregulated p-ERK 3–7 days later (**A**). At 5 or 7 days in culture, p-ERK increased significantly with high-dose (15 μM) of genistein compared with a low-dose (5 μM) or no genistein co-treatment (**B**). Genistein-induced ERK1/2 activation continued for 3 weeks (**C**). Upregulation of p-ERK was not attenuated after the movement of genistein (**D**). While PD98059 (P) and U0126 (U) downregulated ERK1/2 activation in RA-induced differentiation (Fig. 2), they did not block genistein-induced p-ERK upregulation (**E**).

DISCUSSION

Neuronal differentiation is a multi-step developmental process. In this study, we showed that the early phase of neuronal differentiation of human NT-2 cells, which is triggered by aggregation and treatment with RA and genistein, is accompanied by the ERK1/2 activation. Although evidence in multiple cell types indicates the involvement of the MAPKs

signaling cascade in cellular differentiation [Traverse et al., 1992; Alberola-Ila et al., 1995; Gredinger et al., 1998], we demonstrated that ERK1/2 activation together with N-cadherin-mediated cell aggregation is important for human NT-2 neuronal differentiation.

A signaling cascade involved in the triggering of differentiation should be controlled very precisely and should finally act in the nuclear compartment. Indeed, it was recently demonstrated that nuclear translocation of active ERK is crucial for inducing neurite outgrowth, fibroblast transformation, and cell-cycle entry [Brenner et al., 1997; Robinson et al., 1998]. Genistein may have effect at the nuclear levels, such as inhibition of mitogenic signaling pathways [Takano et al., 1994]. Our results agree with previous studies [Reffas and Schlegel, 2000] showing that activated ERK is translocated to the nucleus during the differentiation of P19 cells into neurons, and that cytoplasmic and nuclear ERK activation have distinct kinetics. Our findings support the notion that the functional versatility of the ERK cascade resides not only in the multiple ways in which ERK can be activated, but also in the precise definition of the kinetics and of the cellular compartments in which ERK is active. Here, immunoblotting analysis indicates that ERK1/2 phosphorylation, but neither NF-M expression nor neurite outgrowth, is suppressed by MEK inhibitors. The data imply that the ERK1/2 activation may work during the cell aggregation stage, not during the maturation stage. Intriguingly, although genistein increased RA-induced neuronal differentiation as well as phosphorylated ERK, MEK1/2 inhibitors were unable to block genistein-induced ERK1/2 activation and neurite outgrowth. This may indicate that differential signaling pathways are involved in regulating RA- and genistein-mediated neuronal differentiation. The observations also suggest that genistein is independent of the MEK-ERK signaling cascade. Further, failure of inhibition of genistein-induced ERK activation suggests an alterative pathway caused by long-term treatment of cells with genistein. The fact that the effect of genistein seems irreversible as compared with RA indicates that the ERK1/2 activation has been altered by genistein due to a secondary effect.

In addition to ERK1/2, the p38 MAP/ATF2 system is essential in growth factor-induced neuronal differentiation [Fanger et al., 1997; Morooka and Nishida, 1998]. Although it has been reported [Frey and Singletary, 2003] that genistein activated p38 and inactivated ERK1/2 in mammary epithelial cells, we did not detect p38 expression in NT-2 cells treated with genistein. The administration of p38 inhibitor SB203580 did not affect cluster formation either. These findings suggest that the p38 MAP kinase may not be involved in NT-2 cell cluster formation and further differentiation.

It is well known that N-cadherin and NCAM are important in the development of the nervous system. Generating and maintaining cell aggregation triggers differentiation [Honegger and Richelson, 1976; Maar et al., 1997; Barnea and Roberts, 1999], and treatment with NCAM or other agents is considered to affect process formation. Previous studies showed that genistein had no effect on NCAM and N-cadherin-dependent neurite outgrowth [Williams et al., 1994]. In the present study, N-cadherin expression was elevated along with RA or genistein induction, but downregulated slightly in NT-2 cells treated with the MEK inhibitors. Although coexpression of phospho-ERK and N-cadherin was observed during cellular aggregation and differentiation (data not shown), the interactions between adhersion proteins and MAPK-signaling pathway warrant further investigation. N-cadherin was upregulated in differentiating P19 cells via the Wnt-1 signaling pathway [Gao et al., 2001], the signaling pathway related to cell aggregation remains unclear. NCAM induced neurite outgrowth or neuronal differentiation through the FGF-receptor-mediated signaling pathway and ERK1/2 phosphorylation [Kolkova et al., 2000; Soroka et al., 2002]. In RA- and genistein-induced NT-2 cell differentiation, we did not detect NCAM alteration. It is likely that the selectively stimulatory effect of RA/genistein on NCAM or N-cadherin is cell-type-dependent.

The antiproliferative effects of genistein are mediated by negative cell-cycle regulators such as p21 [Shen et al., 2000], while PCNA acts as a nuclear mitosis protein and S-phase marker. In the present study, we also observed a reduction of PCNA expression and an upregulation of p21 in RA or genistein-treated cells. The effect on PCNA and p21 levels is more obvious in the genistein-treated cells, indicating the influence of genistein on cell proliferation. Activation of the MAPK pathway was linked to the induction of p21 and p27 and cellular differentiation of

HL60 promyelocytic leukemia cells [Das et al., 2000]. Previous studies have also shown that overexpression of p21 induced the expression of monocyte/macrophage-specific markers in the myelomonocytic cell line U937 [Liu et al., 1996]. Moreover, Freemerman et al. [1997] introduced a p21 antisense construct into HL-60 cells and demonstrated that expression of antisense RNA for p21 abolished cellular differentiation. Since PCNA interacts with p21 and Cdk/cyclin to form a quaternary complex [Xiong et al., 1993], the results suggest that increased expression of p21 and downregulation of PCNA may be necessary for the neuronal differentiation.

In conclusion, there are various signaling pathways that function during the neuronal differentiation of NT-2 cells: one may be induced by MAPKs or PTKs, and others induced by cell aggregation or cell–cell interaction. Our findings suggest that RA or genistein may first trigger neuronal differentiation of NT-2 cells by upregulating N-cadherin expression, and then N-cadherin increases the cell–cell interactions necessary for further neuronal differentiation of NT-2 cells. Genistein can enhance RA-induced neuronal differentiation along with ERK1/2 activation; however, a prolonged exposure of differentiating cells to genistein may cause other unknown pathway for maturation. Further experiments will be needed to examine the molecular mechanism underlying this hypothesis.

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