Copper is Required for Retinoic Acid Receptor-Dependent Transcription and Neuronal Differentiation in P19 Embryonal Carcinoma Cells

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Although copper plays a critical role in the embryonic development and differentiation of mammals, the molecular mechanism(s) by which copper deficiency during development leads to embryonic defects has not been sufficiently investigated. In this study, we have focused on the roles of copper in neurogenesis, using mouse embryonal carcinoma P19 cells as a model of neuronal differentiation. In morphological and immunofluorescence studies, we observed that the retinoic acid (RA)-induced neuronal differentiation of P19 cells was suppressed in copper-deficient conditions using a non-permeable copper chelator, BCS. Consistent with this result, minimum amounts of the neuron-specific marker dopamine β-hydroxylase and choline acetyltransferase mRNA were induced in the copper-deficit P19 cells. Furthermore, copper deficiency in P19 cells could suppress the activation of RA receptor (RAR) target genes, such as RARβ2 and cellular retinol binding protein 1 and $p21^{waf1/cip1}$, and RA response element-driven reporter expression by RA. Consequently, our results indicate that intracellular copper is involved in RAR-dependent transcription, which may contribute to explaining the defect of neuronal differentiation in copper-deficit P19 cells.

Key words ——— copper, neuronal differentiation, retinoic acid receptor, P19 cells

INTRODUCTION

Copper is an essential micronutrient for all living organisms; it serves as a redox cofactor for copper-requiring enzymes such as cytochrome c oxidase, dopamine β-hydroxylase, copper, zinc-superoxide dismutase, and lysyl oxidase.[1,2] Copper deficiency is known to cause various physiological disorders resulting from decreased activity of the copper-requiring enzymes. A hereditary disease of copper deficiency in humans, X-linked Menkes disease, is known, in which mutation of genes coding ATP7a, a copper transporter, causes systemic copper deficiency by transport deficits of copper from the gastrointestinal tract into the blood. This disease causes various symptoms that include neurodegenerative disorders with seizures and mental deficiency, kinky hair, hypothermia, hypopigmentation, connective tissue abnormalities, and a fatal outcome.[3–8] Non-hereditary diseases of copper deficiency have been reported to involve anemia, neutropenia, cardiovascular deficits, and bone abnormalities in human patients and experimental animals given copper-deficient diets.[9–13] Recently, deletion of the copper transporter ctr1 gene in mice was reported to cause copper deficiency *in utero*, resulting in early embryonic death.[14,15] Additionally, copper deficiency during pregnancy has been shown to lead to early embryonic death or structural and functional abnormalities in the fetus.[16,17] It has been therefore demonstrated that copper plays an extremely important role in embryonic development and differentiation.

The central nervous system is susceptible to copper deficiency because of high copper content. Neurodegeneration in Menkes patients is hypothesized to result from decreased production of energy and catecholamines and increased production of reactive oxygen species due to decreased activities of the copper-requiring enzymes in the brain as a result of copper deficiency. Interestingly, the onset of neurological symptoms in patients occurs before birth[13]; therefore, decreased activity in the cop-

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per-requiring enzymes during development can be expected to especially affect neural development and growth.

In P19 cells, a mouse embryonal carcinoma cell line, differentiation into neural cells is induced by all-*trans* retinoic acid (RA; active form of vitamin A) and the formation of cellular aggregates.[18] These cells are widely used for studies of neural developmental processes such as neurite outgrowth and differentiation. Here, we study the effects of copper-deficient culture of P19 cells on neuronal differentiation using bathocuproine disulfonic acid (BCS), which is a cell-impermeable and copper-specific chelator. The results show that copper deficiency in P19 cells suppresses RA-induced neuronal differentiation and also suppresses RA and its receptor-mediated gene expression. Our results suggest that copper may be an essential factor for the initial stages of neuronal differentiation.

MATERIALS AND METHODS

Cell Culture, Neuronal Differentiation, and BCS Treatment ——— Mouse P19 embryonal carcinoma cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University, and maintained at 37°C in a humidified 5% CO_2 atmosphere in α-minimum essential medium (JRH Bioscience, Lenexa, KS, U.S.A.), supplemented with 10% fetal bovine serum, 100 unit/ml streptomycin, and 100 μg/ml penicillin. To induce neuronal differentiation, P19 cells were grown as an aggregate in 15-ml conical tubes in the presence of 0.5 μM all-*trans* RA (Wako Pure Chemical Industries, Osaka, Japan). After 3 days of aggregation, the cells were dissociated by pipetting and plated on cell culture dishes in the absence of RA. A non-permeable copper(I) chelator, BCS (50 μM; Wako Pure Chemical Industries), was added to the culture medium of P19 cells at the same time as treatment with RA.

Plasmid Constructs ——— The promoter-less β-galactosidase reporter vector was constructed according to Uetsuki *et al*. with some modification.[19] The β-galactosidase cassette was obtained by HindIII and BamHI digestion of pSV-β-galactosidase (Promega, Madison, WI, U.S.A.) and cloned into the HindIII and BamHI sites of pBluescript (Stratagene, La Jolla, CA, U.S.A.). The resultant construct was named pBSZ. To make a neuron-specific reporter construct (pNFZ) and an RA-response element (RARE)-driven reporter construct (pRAREZ), the 5′-flanking region (–1574 to +70) of the mouse neurofilament light (NF-L) gene and the RARE-containing the 5′-flanking region (–88 to +112) of the mouse RA receptor β2 gene were PCR amplified using specific primers with a HindIII site from mouse genomic DNA and then subcloned into the HindIII site of pBSZ.

Establishment of Stable Cell Lines ——— P19 cells were cotransfected with pNFZ or pRAREZ and a neomycin-resistant plasmid (pRcCMV2) at a ratio of 10 : 1 using FuGENE6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN, U.S.A.). Twenty-four hours later, transfection cultures were subcultured at a 1 : 10 dilution into a complete medium containing 750 μg/ml G418 and maintained for 8–10 days. The resultant G418-resistant clones were screened by β-galactosidase assay, and the positive clones were obtained and named P19NF cells and P19RA cells, respectively.

Immunofluorescence Staining ——— P19 cells were fixed with 3.7% formalin in the culture medium for 30 min at room temperature. Cells were permeabilized with 0.3% triton X-100 in PBS with 5% horse serum for 20 min and incubated with primary antibody against βIII-tubulin (1 : 2000; Promega) in PBS containing 0.5% horse serum at 4°C overnight. After rinsing in PBS, the samples were incubated with secondary antibody conjugated to Alexa Fluor 488 (1 : 800; Invitrogen, Carlsbad, CA, U.S.A.) in PBS for 2 hr at room temperature. Immunofluorescence was visualized using a fluorescent Olympus microscope (IX70; Olympus, Tokyo, Japan).

Semi-Quantitative RT-PCR ——— Total RNA was extracted from cells using Trizol reagent (Invitrogen). For each sample, 2 μg RNA was reverse-transcribed into cDNA in a final volume of 20 μl with 50 pmol oligo(dT) and 50 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Nippon Gene, Tokyo, Japan) for 60 min at 37°C. PCR was performed in a final volume of 20 μl with 1 μl RT product for cDNA amplification. The PCR mixture contained 20 pmol each of forward and reverse primers and 1 U *Taq* polymerase (Promega). Primer sequences and PCR conditions are listed in Table 1. The PCR products were separated on 2% agarose gels and visualized by ethidium bromide.

β-Galactosidase Assay ——— β-galactosidase activity was determined using the β-galactosidase enzyme assay system (Promega) according to the manufacturer's instructions and normalized for total protein.

Table 1. RT-PCR Information: Primer Sequences and PCR Conditions

Genes	Primer sequences (5′–3′)	Anealing		Cycles
		°C	sec	
ChAT	F: ATGCCTATCCTGGAAAAGGTCCC	65	30	35
	R: ACCTCACTGAGACGGCGGAAATAA			
DBH	F: TGAAGTCTGAGGTCCCCACT	60	30	25
	R: CATCTGGGTGCACTTGTCTG			
Nestin	F: AGTGAATGAGGCCTTCGAGA	65	30	25
	R: CTGGGTTCCCTGTTCTGTGT			
c-jun	F: ACGACCTTCTACGACGATGC	60	30	23
	R: AGTTGCTGAGGTTGGCGTAG			
Mash1	F: AGAGCTCTGGCAAGATGGAG	60	30	28
	R: GAACCCGCCATAGAGTTCAA			
Neurogenin1	F: TCTGATCTCGACTGCTCCAG	65	30	37
	R: GAAAGGAGAAAAGGGGATCG			
NeuroD1	F: TCAACCCTCGGACTTTCTTG	60	30	29
	R: GCATTAAGCTGGGCACTCAT			
RARβ2	F: TTGCACACACTCACCACCTT	55	30	30
	R: CGAGCTCCTCAGAGCTGGTA			
CRBP1	F: ATCACCCGGAGTGCATTG	60	30	23
	R: CTTGCAGATCACACCCTCAG			
$p21^{waf1/cip1}$	F: CGGTCCCGTGGACAGTGAGCAG	65	30	20
	R: GTCAGGCTGGTCTGCCTCCG			
β-actin	F: TGTTACCAACTGGGACGACA	65	30	18
	R: TCTCAGCTGTGGTGGTGAAG			

PCR conditions: denaturation at 94°C for 30 sec; annealing as listed in the table; extension at 72°C for 30 sec. F: forward, R: reverse.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA) ——— P19 cells were washed twice with PBS and lysed with lysis buffer (0.2% NP-40, 10 mM HEPES-KOH, pH 7.4, 2 mM $MgCl_2$, 15 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The lysates were centrifuged at 800 $\times$ *g* for 30 sec. The pellets were washed with sucrose buffer (250 mM sucrose, 10 mM HEPES-KOH, pH 7.4, 2 mM $MgCl_2$, 15 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF). The resulting pellets were resuspended in high-salt buffer (400 mM KCl, 50 mM HEPES-KOH, pH 7.9, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF), vortexed using a microtube mixer for 30 min at 4°C, and centrifuged at 15000 rpm for 20 min. The supernatants were used as nuclear extracts. For the EMSA, the consensus AP-1 oligonucleotide probe (5′-AGCTCGCTTGATGAGCTAGCCGGAA-3′) was end-labeled with Klenow enzyme in the presence of [α-^{32}P] dCTP. The binding reaction mixture contained 3 μg of nuclear extract, 2 μg poly(dI–dC), 40 mM Tris–HCl, pH 7.9, 100 mM KCl, 4 mM $MgCl_2$, 2 mM EDTA, 0.2% NP-40, 20% glycerol, 0.2 mM DTT, and 100000 cpm of end-labeled DNA fragments in a total volume of 20 μl. After 30 min incubation at room temperature, the samples were loaded onto a 4% polyacrylamide gel and run in a 0.5 $\times$ TBE electrophoresis buffer. These gels were dried and exposed to X-ray film at –80°C.

RESULTS

Copper Deficiency Suppresses RA-Induced Neuronal Differentiation of P19 Cells

To investigate whether intracellular copper is involved in the neuronal differentiation of P19 cells, we treated them in differentiation medium with BCS, a non-permeable copper chelator, at a concentration of 50 μM. Neurons were identified by morphology and by staining with the antibody against neuron-specific tubulin (βIII-tubulin) in control P19 cells at 5 days after the initiation of differentiation (Fig. 1A). Conversely, in P19 cells cotreated with BCS for 5 days, βIII-tubulin-positive differentiated neurons were hardly observed (Fig. 1A). Furthermore, we tested the effects of BCS on the expression of neu-

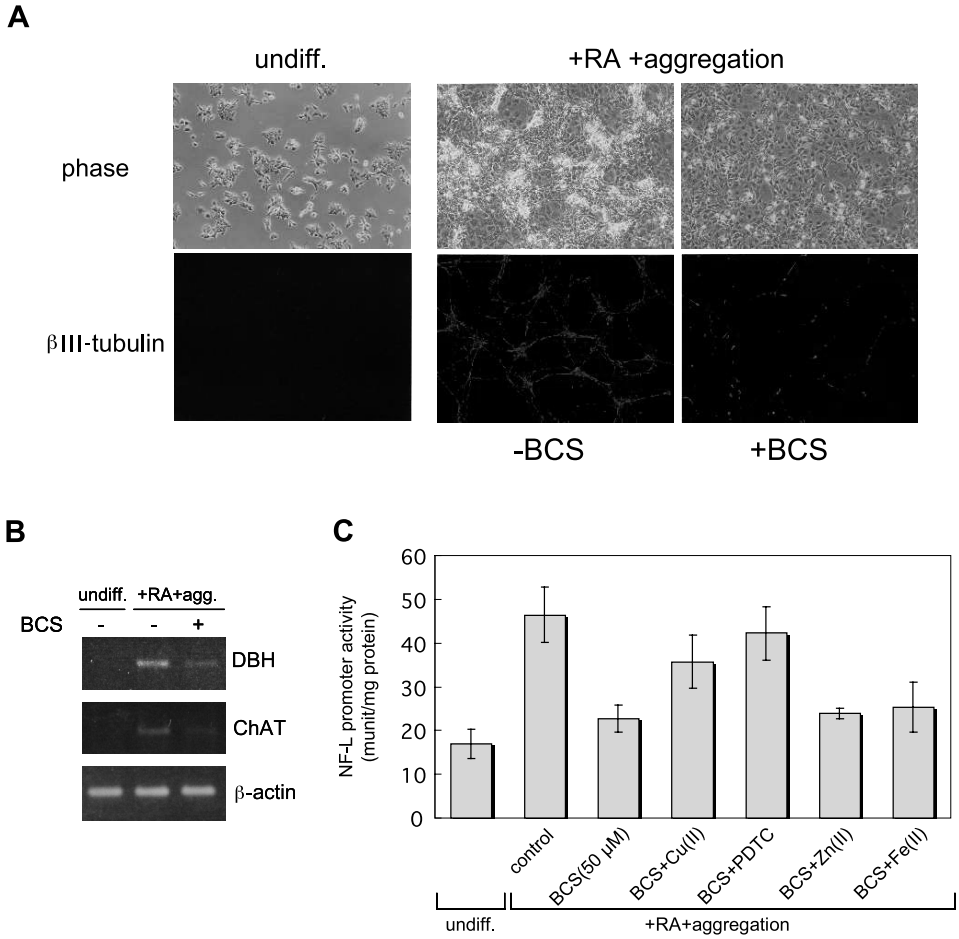

Fig. 1. Suppression of P19 Neuronal Differentiation by Copper Deficiency

(A) P19 cells were treated with RA and cellular aggregation in the presence or absence of BCS. After 3 days of incubation, the cells were plated onto cell culture dishes and incubated for 48 hr, and then were fixed and immunostained for βIII-tubulin. (B) P19 cells were treated as described above. After 3 days of incubation, the expression of DBH, ChAT, and β-actin was analyzed by semi-quantitative RT-PCR. (C) P19NF cells bearing a neuron-specific reporter gene were treated as described above, and $CuCl_2$, PDTC, $ZnCl_2$, or $Fe(NH_4)_2(SO_4)_2$ was added concomitantly in the presence of BCS. After 3 days of incubation, the cells were plated onto cell culture dishes and incubated for 24 hr and were then harvested for the β-galactosidase assay. Values are the mean ± S.D., $n = 3$.

ron-specific genes such as dopamine β-hydroxylase (DBH) and choline acetyltransferase (ChAT) by semi-quantitative RT-PCR analysis. As expected, the expression of these genes was strongly induced by RA treatment with cellular aggregation for 3 days (Fig. 1B). In contrast, minimal amounts of ChAT and DBH were induced in the BCS-treated P19 cells (Fig. 1B). For quantitative analysis of neuronal differentiation, we constructed a neurofilament-L promoter-driven β-galactosidase reporter plasmid (pBZNF) and generated stable clones from P19 cells bearing the reporter gene (P19NF). The β-galactosidase activity in differentiated P19NF cells (4 days) was markedly increased compared to that in undifferentiated P19NF cells (Fig. 1C). P19NF cells cotreated with BCS showed hardly any increase in β-galactosidase activity at 4 days after RA treatment with cellular aggregation for 3 days (Fig. 1C). We next confirmed whether the suppression of neuronal differentiation by BCS is caused by copper deficiency. The effect of BCS was blocked by the addition to the medium of $CuCl_2$ (1 μM) but was not blocked by the addition of $ZnCl_2$ (100 μM) or $Fe(NH_4)_2(SO_4)_2$ (1 μM) (Fig. 1C). It is thought that the reduction of copper(II) to copper(I) is an important process in CTR1-mediated copper uptake.[1)] A non-permeable copper(I) chelator, BCS, binds to copper(I) in the culture medium and consequently can induce copper deficiency. We showed that the suppression of neuronal differentiation by BCS was restored by the addition of a low level of $Cu(II)Cl_2$. Since it has been reported that a copper(II)-histidine complex was incorporated into cell culture,[20,21)] we suggest that copper(II) rapidly binds to small mol-

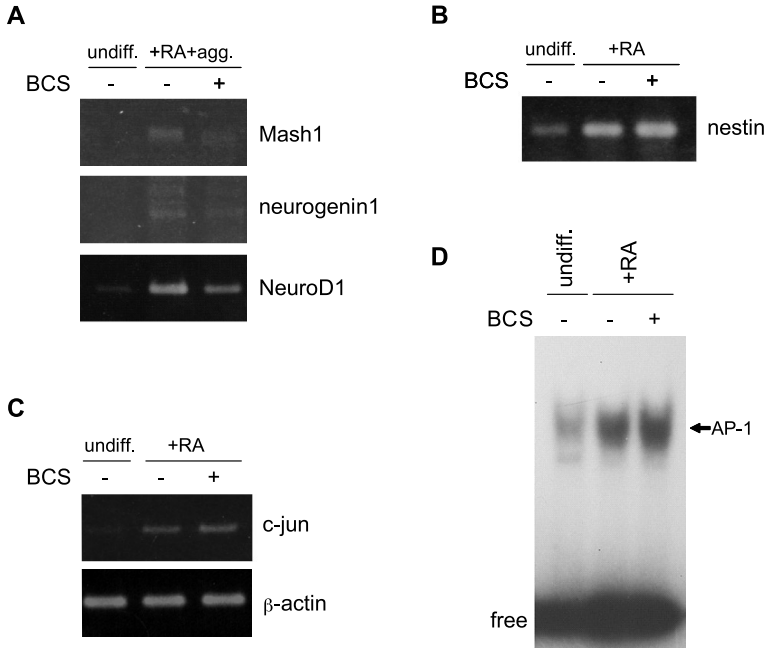

Fig. 2. Expression of Differentiation-Related Genes under Copper Deficiency Conditions

(A) P19 cells were treated with RA and cellular aggregation in the presence or absence of BCS. After 3 days of incubation, the cells were cultured onto cell culture dishes and incubated for 24 hr, and then the expression of Mash1, neurogenin 1, and NeuroD1 was analyzed by semi-quantitative RT-PCR. (B–D) P19 cells were treated with RA without aggregation in the presence or absence of BCS. After 3 days of incubation, the expression of nestin (B) and c-jun (C) was analyzed by semi-quantitative RT-PCR, and the binding activity of AP-1 in nuclear extracts was analyzed by EMSA (D).

ecules such as histidine in the culture medium and is incorporated into RA-treated aggregated P19 cells without influence by BCS. A cell-permeable copper(II) chelator, pyrrolidine dithiocarbamate (PDTC), has been shown to bind to copper(II) in the culture medium and mediate the accumulation of copper into cells.[22–24] We observed that the effect of BCS was also blocked by the addition of PDTC (10 μM) (Fig. 1C). Moreover, we confirmed that the decreased activity (26% of control) of copper, zinc-superoxide dismutase induced by BCS was restored by the addition of $CuCl_2$ (66% of control) or PDTC (89% of control) (data not shown). These results indicate that the suppressive effect of BCS on neuronal differentiation is dependent upon copper deficiency in P19 cells.

The Expression Change of the Differentiation-Related Genes is Affected or not Affected by Copper Deficiency

Neurogenesis is promoted by basic helix-loop-helix (bHLH) transcription factors such as Mash1, neurogenin, and NeuroD.[25,26] We examined whether these transcription factors could be induced during neuronal differentiation of P19 cells under copper-deficient conditions. In RT-PCR experiments, Mash1, neurogenin 1, and NeuroD1 were strongly induced by RA treatment and aggregation for 3 days, whereas they were only slightly induced in the presence of BCS (Fig. 2A). Nestin, a neural stem cell marker, is reported to also be induced by RA in P19 cells.[27] We therefore examined the effect of BCS on this induction. As shown in Fig. 2B, there was no effect of BCS in P19 cells on the induction of nestin by RA. The transcription factor c-Jun, which is a component of the AP-1 transcription factor, is induced during RA-induced neuronal differentiation of P19 cells, and the DNA binding activity of AP-1 is induced in the nuclear extract of differentiated P19 cells.[28] Overexpression of c-Jun in P19 cells has been shown to lead to differentiation into endoderm- and mesoderm-like cells.[29] To investigate the effect of copper deficiency on c-jun expression by RA, P19 cells in monolayer culture were treated with RA in the presence or absence of BCS for 3 days, and the expression level of c-jun was determined by semi-quantitative RT-PCR analysis. As shown in Fig. 2C, c-jun expression was enhanced by RA in the absence of BCS. In the case of BCS-cotreated cells, a similar increase in c-jun was observed. Moreover, morphological changes of P19 cells induced by RA without aggregation were not affected by the addition of

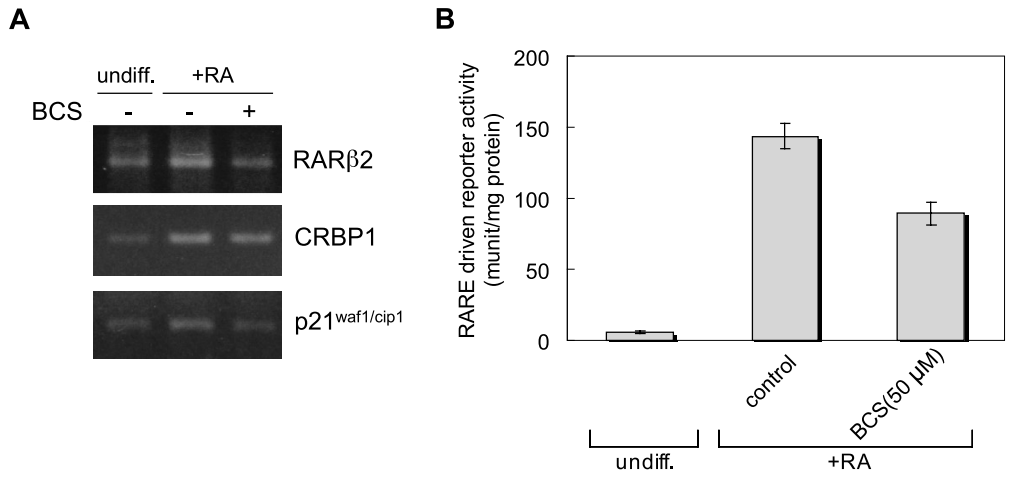

Fig. 3. Suppression of RA and its Receptor-Mediated Gene Activation by Copper Deficiency

(A) P19 cells were treated with RA without aggregation in the presence or absence of BCS. After 3 days of incubation, the expression of RARβ2, CRBP1, and p21$^{waf1/cip1}$ was analyzed by semi-quantitative RT-PCR. (B) P19RA cells bearing the RARE-driven reporter gene were treated as described above. After 3 days of incubation, the cells were harvested for β-galactosidase assay. Values are the mean ± S.D., $n = 3$.

BCS (data not shown). In EMSA experiments, RA increased the binding activity of AP-1 in a nuclear extract of P19 cells and also increased the activity in the presence of BCS (Fig. 2D).

Copper Deficiency Suppresses RA and its Receptor-Mediated Gene Activation

The effects of RA are known to be mediated by retinoic acid receptors (RARs), which belong to the nuclear receptors. The receptors normally act as ligand-inducible transcriptional factors by binding to RARE present in target genes. We investigated the effect of copper deficiency on the expression of RAR-mediated gene activation in P19 cells. As shown in Fig. 3A, the expression of RAR-target genes such as RARβ2, cellular retinol binding protein 1 (CRBP1), and p21$^{waf1/cip1}$ [30–34] was induced by RA without cellular aggregation, whereas it was induced only slightly or not at all in the presence of BCS. To further confirm that the suppression of induction of RA-target genes by copper deficiency is involved in RAR and RARE-mediated transcription, we constructed the reporter gene driven by the RARE-containing promoter of RARβ2 (pBZRA) and generated stable clones from P19 cells bearing the reporter gene (P19RA). P19RA cells in monolayer culture were treated with RA in the presence or absence of BCS for 3 days and harvested for β-galactosidase assay. The β-galactosidase activity in RA-treated control cells was markedly increased compared to that in undifferentiated P19RA cells (Fig. 3B). In BCS-cotreated P19RA cells, the β-galactosidase activity was approximately 60% of that in control cells (Fig. 3B). The results indicate that intracellular copper is involved in RAR-mediated transcriptional activation.

Cytochrome *c* Oxidase Inhibitor does not Suppress RAR-Dependent Transcription

Copper deficiency causes the decreased production of energy resulting from the decreased activity of cytochrome *c* oxidase (CCO).[1] It is therefore suggested that inhibition of CCO in P19 cells may cause suppression of RAR-dependent transcription and neuronal differentiation. To examine this hypothesis, P19RA cells were treated with RA in the presence of a CCO inhibitor, sodium azide (NaN_3), and β-galactosidase activity was determined 3 days after RA treatment. As shown in Fig. 4A, β-galactosidase activity was induced by RA in the presence of 10 μM NaN_3, as well as in control cells. The activity in P19RA cells in the presence of 100 μM NaN_3 was slightly higher than in control cells. Next, P19NF cells were treated by RA with aggregation in the presence of NaN_3, and β-galactosidase activity was determined 4 days after RA treatment. The increased activity of RA with aggregation was slightly suppressed by addition of 100 μM NaN_3 (Fig. 4B). The NaN_3-added cells were observed to accompany significant cell death, while the addition of BCS could not promote cell death (data not shown). These results suggest that the effects of BCS on the neuronal differentiation of P19 cells may not be mediated only by decreased CCO activity.

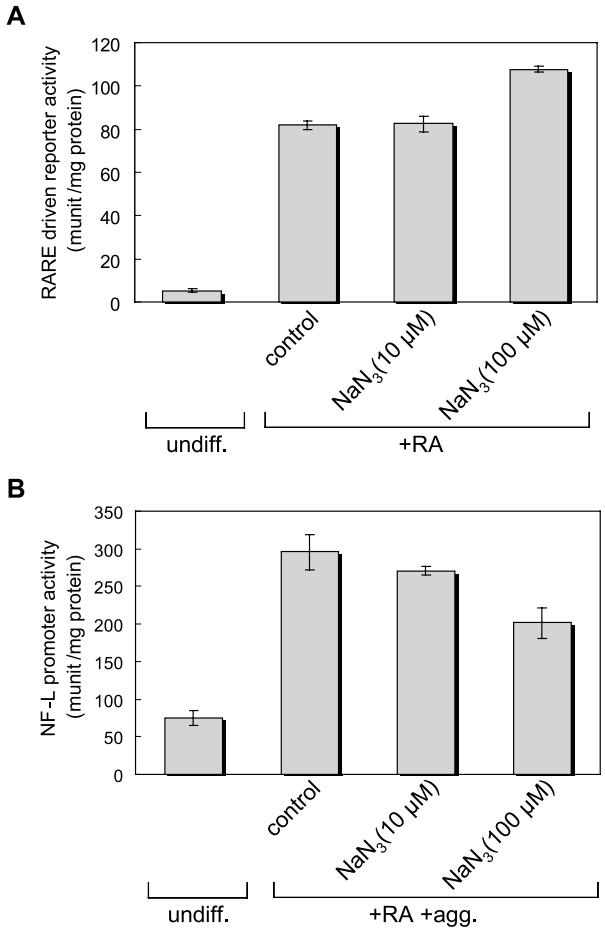

Fig. 4. Cytochrome *c* Oxidase Inhibitor does not Affect RAR-Dependent Transcription

(A) P19RA cells were treated with RA without aggregation in the presence or absence of NaN_3 (10, 100 μM). After 3 days of incubation, the cells were harvested for β-galactosidase assay. (B) P19NF cells were treated with RA and aggregation in the presence or absence of NaN_3 (10, 100 μM). After 3 days of incubation, the cells were plated onto cell culture dishes and incubated for 24 hr and then were harvested for β-galactosidase assay. Values are the mean ± S.D., $n = 3$.

DISCUSSION

Several studies have shown that copper plays a critical role in the embryonic development of mammals.[14–17] In this study, we have focused on the roles of copper in neurogenesis and investigated them using P19 cells as a model of neuronal differentiation. We show here that the neuronal differentiation of P19 cells is suppressed in copper-deficient conditions using the non-permeable copper chelator BCS (Fig. 1), which may be correlated with the fact that a deficit of copper in patients with Menkes disease and experimental mice can lead to defective neuronal development in the fetus and newborn. Moreover, as shown in Fig. 1, we found that copper deficiency in P19 cells suppresses not only the induction of mature neuronal markers including βIII-tubulin, DBH, and ChAT, but also the induction of proneural bHLH genes during neuronal differentiation. Interestingly, copper deficiency in P19 cells could not affect the induction of the immediate early response gene c-jun and the neural stem cell-marker nestin by RA (Fig. 2). Therefore, we speculate that the mechanism by which copper deficiency suppresses the neuronal differentiation of P19 cells is by affecting the particular signaling pathway in differentiation, rather than by reducing cellular energy production due to CCO inactivation arising from copper deficiency. To examine this hypothesis, we investigated the effect of copper deficiency on RAR target gene activation. As shown in Fig. 3, copper deficiency in P19 cells can suppress the activation of RAR target genes, such as RARβ2, CRBP1, and $p21^{waf1/cip1}$, and also RARE-driven reporter expression by RA. In contrast, we observed that NaN_3, a CCO inhibitor, cannot suppress this reporter expression (Fig. 4). These findings suggest that the suppression of RA-induced neuronal differentiation of P19 cells by copper deficiency results from the reduction of gene activation through RARE in the promoters of target genes.

Why did copper deficiency not affect the induction of c-jun and nestin by RA? It is reported that there is a nuclear receptor-independent pathway for the action of RA during RA-induced F9 mouse carcinoma cell differentiation.[35] Moreover, the inhibitory effect of RA on oxidant-induced apoptosis of mesangial cells has been shown to be mediated in part through a nuclear receptor-independent mechanism.[36] We therefore suggest that the expression of c-jun and nestin during differentiation of P19 may be mediated though a RAR-independent mechanism, and thus our results indicate that copper deficiency may affect RAR-dependent but not RAR-independent mechanisms in the RA signaling pathway.

It is known that RA plays a critical role in early embryonal development including that of the neural system in mammals.[37,38] Mice homozygous for disruption of the retinaldehyde dehydrogenase type 2 gene, which encodes an enzyme for the synthesis of RA, have an abnormality at embryonic day 8.5 (E8.5) and die by E10.5.[39] Interestingly, copper deficiency caused by disruption of the CTR1 gene in mice has been shown to cause an embryonic abnormality at E7.5 and death by E10.[14,15] Furthermore, it has been reported that the disruption of the COX17p gene that identified a copper chaperone for CCO leads to CCO deficiency and results in embryonic lethality between

E8.5 and E10.[40] Our results show that copper deficiency causes a defect of action of RA in embryonal carcinoma cells. Although it is possible that the embryonic defect in CTR1-deficient mice is caused by CCO inactivation, it is necessary to clarify whether the defect of action of RA arising from copper deficiency is implicated, at least in part, in this embryonic defect.

In conclusion, this study demonstrates that copper is required for the action of RA through RAR during neuronal differentiation of P19 cells. Moreover, it is possible that the defect of action of RA caused by copper deficiency affects not only neurogenesis but also various aspects of normal embryogenesis in mammals because of the RA requirement for embryogenesis. Further studies should be designed to elucidate their relationship to the pathogenesis of copper deficiency diseases such as Menkes disease during embryonic development and to discover the copper-required protein(s) involved in the RAR signaling pathway.

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