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Expression of Retinoid Receptors During the Retinoic Acid-Induced Neuronal Differentiation of Human Embryonal Carcinoma Cells

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Abstract: Retinoic acid (RA), a derivative of vitamin A, is essential for normal patterning and neurogenesis during development. Until recently, studies have been focused on the physiological roles of RA receptors (RARs), one of the two types of nuclear receptors, whereas the functions of the other nuclear receptors, retinoid X receptors (RXRs), have not been explored. Accumulating evidence now suggests that RXRα is a critical receptor component mediating the effects of RA during embryonic development. In this study, we have examined the expression profiles of RXRα and RARs during the RA-induced neuronal differentiation in a human embryonal carcinoma cell line, NT2. Distinct expression profiles of RXRα, RARα, RARβ, and RARγ were observed following treatment with RA. In particular, we found that RA treatment resulted in a biphasic up-regulation of RXRα expression in NT2 cells. The induced RXRα was found to bind specifically to the retinoid X response element based on gel mobility retardation assays. Furthermore, immunocytochemical analysis revealed that RXRα expression could be localized to the somatoaxonal regions of the NT2 neurons, including the tyrosine hydroxylase- and vasoactive intestinal peptide-positive neurons. Taken together, our findings provide the first demonstration of the cellular localization and regulation of RXRα expression in NT2 cells and suggest that RXRα might play a crucial role in the cellular functions of human CNS neurons. **Key Words:** Retinoic acid—Neuronal differentiation—Retinoid receptors—NT2—Embryonal carcinoma.
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Retinoic acid (RA), a derivative of vitamin A, is important for the maintenance of normal growth and development (Morriss-Kay and Sokolova, 1996). It is a natural morphogen that determines anteroposterior axial patterning as well as neuronal differentiation (Conlon, 1995; Papalopulu and Kintner, 1996). RA induces the differentiation of many cell types, including neuronal precursor cells and epithelial cells (De Luca, 1991; Cheung and Ip, 1998). Clinically, it has been used as an effective therapeutic agent for the treatment of acute promyelocytic leukemia (Warrell et al., 1991). Based on the importance of RA in the embryogenesis of vertebrates and its potential use as therapeutic agents for cancer cells, many of the studies have been focused on elucidating the mechanism of action of RA.

RA acts on target cells via two classes of nuclear receptors that belong to the nuclear hormone receptor family. The RA receptors (RARα, RARβ, and RARγ) bind to both all-*trans* (*t*)-RA and 9-*cis* (9*c*)-RA, whereas the retinoid X receptors (RXRα, RXRβ, and RXRγ) bind only to 9*c*-RA (Chambon, 1996). The retinoid signal is transduced by the formation of RXR/RAR heterodimers, which bind to the RA response elements (RAREs) to activate the gene expression of RA-responsive genes (Kastner et al., 1997). These RARs have been demonstrated to be important in tumor differentiation, including myeloid leukemia cells and human embryonal carcinoma (EC) cells (Collins et al., 1990; Simeone et al., 1990). Whereas RARα is essential for the responsiveness of HL-60 cells to *t*-RA, RARγ overexpression can restore the RA-induced terminal mesenchymal differentiation of RA-resistant human EC cells (Moasser et al., 1995). Although extensive studies have been focused on the roles of RARs, little is known of the importance of the RXRs, especially during RA-induced neuronal differentiation.

Although RXR/RAR heterodimers bind more effectively to the RAREs, RXRs alone can also form homodimers that bind to the retinoid X response elements

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Abbreviations used: EC, embryonal carcinoma; MAP2, microtubule-associated protein 2; NF-M-P, phosphorylated neurofilament 160 kDa; NS, nonsense; PBS, phosphate-buffered saline; RA, retinoic acid; RAR, RA receptor; RARE, RA response element; RXR, retinoid X receptor; RXRE, retinoid X response element; TH, tyrosine hydroxylase; TX, Triton-X; VIP, vasoactive intestinal peptide.

(RXREs) with high affinity (Zhang et al., 1992). Thus, it has been proposed that RXRs can acquire different functional roles by homodimerization of the receptors. The diverse but unique expression of RXRs also suggests the possible involvement of RXRs in various physiological functions as well as in different aspects of development and neurogenesis (Mangelsdorf et al., 1992). The importance of RXRs is further exemplified by recent knockout studies (Kastner et al., 1997). For example, RXR$\alpha^{-/-}$ mice died at early embryonic stages and exhibited cardiac defects characteristic of the vitamin A deficiency syndrome (Sucov et al., 1994). Although accumulating evidence supports the involvement of RXRα in muscle development, similar roles in neuronal differentiation remain to be elucidated. A low level of expression of RXRα transcript was previously detected in the CNS of the mouse embryo (Mangelsdorf et al., 1992; Dolle et al., 1994), and a recent study on the expression of RXRα revealed its association with the rostrocaudal patterning of the neural tube (Hoover and Glover, 1998). Although several studies confirmed the expression of RXRα in the developing CNS and proposed the involvement of RXRα in neural development, the role of RXRα during the neuronal differentiation of the neuronal precursor cells has not been explored.

In the present study, we employed a human cell line, NT2 cells, as a model system to examine the role of RXRα during RA-induced neuronal differentiation in the human CNS. NT2 cells have been used extensively as model systems for studying both neurodegenerative diseases such as Alzheimer's disease and the molecular mechanisms of RA actions (Cheung et al., 1996; Maerz et al., 1998). When treated with RA for >4 weeks, NT2 cells differentiate into postmitotic CNS neurons (Pleasure et al., 1992). The RA-induced neuronal differentiation of NT2 cells requires continuous exposure to *t*-RA (Andrews, 1984), suggesting the possible involvement of RA at the later stages of RA-induced neuronal differentiation of NT2 cells. In this study, we examined both the transcript and the protein expression of RXRα and RARs during prolonged treatment with RA in NT2 cells as well as the subcellular localization of RXRα. These findings provide potential insights into our understanding of the functional roles of RXRα during RA-induced neuronal differentiation.

MATERIALS AND METHODS

Cell culture

NT2 cells were cultured in Opti-MEM I medium (Gibco) supplemented with 5% fetal bovine serum (Gibco) as previously described (Pleasure et al., 1992; Cheung et al., 1997). The cells were differentiated with either *t*-RA (2 or 10 μM; Sigma) or 9*c*-RA (2 or 10 μM; Sigma) in Dulbecco's modified Eagle's medium (high glucose formulation) supplemented with 10% fetal bovine serum. NT2 neurons were prepared using either the monolayer approach or the cell aggregation method as described (Pleasure et al., 1992; Cheung et al., 1999).

Immunocytochemical analysis

NT2 neuron cultures were fixed with 2.5% paraformaldehyde (Fluka) in phosphate-buffered saline (PBS). Cells were blocked with 4% normal serum (Vector Laboratories) in PBS containing 0.25% Triton-X (TX; Roche Molecular Biochemicals) and 2% bovine serum albumin (Sigma) and were incubated overnight at 4°C with primary antibodies diluted in antibody diluent (4% normal serum, 0.25% TX, and 2% bovine serum albumin). Incubation with secondary antibodies was performed at room temperature for at least 2 h. Polyclonal rabbit antisera detecting different RARs (α, β, γ) and RXRs (α, β, γ) were obtained from Santa Cruz; polyclonal rabbit anti-tyrosine hydroxylase (anti-TH) was obtained from Chemicon. Rabbit polyclonal antibody specific for vasoactive intestinal peptide (VIP; Sigma) and monoclonal mouse antisera specific for phosphorylated neurofilament 160 kDa (NF-M-P; Zymed) and microtubule-associated protein 2 (MAP2; Zymed) were used in the present study. Alexa488 or Alexa568-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (Molecular Probes) were used as secondary antibodies. Positive signals were not obtained using secondary antibodies alone; specificity of the signals detected by the RXRα antibody was confirmed when the immunoreactivity was blocked following preabsorption with the specific peptide (data not shown).

Protein extraction and western blot analysis

Cells were washed with ice-cold PBS followed by ice-cold PBS supplemented with 1 m*M* sodium orthovanadate and Complete protease inhibitor cocktail (Complete; Roche Molecular Biochemicals). Cells were then lysed with lysis buffer [50 m*M* Tris-Cl (pH 8), 150 m*M* NaCl, 1% TX, 1× aprotinin (Sigma), 1 m*M* phenylmethylsulfonyl fluoride, 1 m*M* sodium orthovanadate, and 1× Complete] at 4°C for 15 min. Lysates were collected and centrifuged to remove cell debris. Protein assays were performed with the Bio-Rad Protein Assay Kit. Protein samples (typically 20 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 10% resolving gel using a Bio-Rad minigel apparatus and then electrotransferred to nitrocellulose membrane (Amersham-Pharmacia Biotech). After blocking at room temperature for 1 h using Tris-buffered saline/Tween containing 5% nonfat milk, membranes were incubated overnight at 4°C with primary antibodies in Tris-buffered saline/Tween containing 5% nonfat milk. Membranes were then incubated with 1:1,500 horseradish peroxidase-conjugated secondary antibodies (Amersham-Pharmacia Biotechnology) for 1 h at room temperature. After three washes with Tris-buffered saline/Tween, immunoreactive bands were detected using an ECL kit (Amersham-Pharmacia Biotechnology) and visualized by Fuji x-ray film.

RNA preparation and RT-PCR analysis

Total RNA was prepared using Trizol reagent (Gibco). Equal amounts (2 μg) of total RNA were prepared from NT2 cells after treatment with RA for various periods (1–21 days) and used for RT with Superscript II reverse transcriptase (Gibco). One-twentieth of the reaction was amplified using HIFI *Taq* DNA polymerase (Gibco) with specific primers. For RXRα, gene expression was confirmed by using different number of PCR cycles, and Southern blot analysis was performed using specific human RXRα cDNA probes.

Gel retardation assays

Gel retardation assays were performed as described with minor modifications (Hope and Struhl, 1987). In brief, nuclear

TABLE 1. *Primers used for RT-PCR analysis of transcript expression of RXR isoforms in NT2 cells*

Specificity	Downstream primer(s)[a]	Annealing temperature (°C)[b]	Product size (bp)
RXRα (full length)	5′ atggacaccaaacatttc 5′ ctaagtcatttggtgcggcgcctccagcatctcc	52	1,388
RXRα (partial)	5′ ctgtttgtcggctgcttg 5′ cctcaatggcgtcctcaa	54	490
RXRβ (partial)	5′ cccgcccttcctccctcagc 5′ gcccttgcaaccctcacagc	58	664
RXRγ (partial)	5′ ccacactggctctacatcc 5′ tccttatcgtcctcttgaa	50	446

[a] The upper primers were the downstream primers and the lower primers were the upstream primers used for the amplification.
[b] Annealing temperature was determined experimentally (data not shown).

extracts prepared from NT2 cells that were treated with *t*-RA for 5 days were incubated with radioactively labeled oligonucleotides in 1× binding buffer containing 20 m*M* Tris-HCl, 50 m*M* KCl, 3 m*M* $MgCl_2$, 1 m*M* EDTA, 100 μg/ml gelatin, and 1 μg of poly(dI–dC) at pH 7.4. Incubation was performed at room temperature for 20 min. The reaction mixtures were electrophoresed in a 5% nondenaturating polyacrylamide gel containing 0.5× Tris/borate/EDTA for 3 h. The probe used included the oligonucleotide RXRE (5′-AGC TGT CAC AGG TCA CAG GTC ACA GGT CAC AGT TCA AGC T) encoding the RXRE of the rat cellular retinol binding protein. It was previously demonstrated that the human RXRα could bind to this oligonucleotide in gel retardation assay (Mangelsdorf et al., 1991). As a control, the oligonucleotide RARE (5′-AGC TTA AGG GTT CAC CGA AAG TTC ACT CGC ATA GCT) containing the RARE from RARβ promoter (Sucov et al., 1990) was used. The nonsense oligonucleotide NS (5′-AGC TCT CTG TTC TCC TGG TGT TCT GAA GCT) was used as a negative control in the gel retardation assays. In the competition assays, in addition to the unlabeled RXRE, RARE, and NS, oligonucleotides encoding the consensus sequences of different transcription factors such as AP2 (5′-GAT CGA ACT GAC CGC CCG CGG CCC GT) and SP1 (5′-ATT CGA TCG GGG CGG GGC GAG C) were also employed.

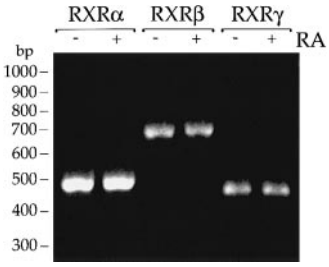

FIG. 1. RT-PCR analysis of RXR isoforms in NT2 cells. Total RNA was collected from NT2 cells either cultured in normal medium (−) or treated with *t*-RA (10 μ*M*) for 14 days (+). An equal amount (2 μg) of total RNA was reverse-transcribed using SuperScript II RNaseH$^-$ RT (Gibco). Amplification by PCR was performed using Platinum *Taq* DNA polymerase (Gibco) in a Robocycler (Stratagene) for 40 cycles. Amplified DNA products representing RXRα, RXRβ, and RXRγ were separated on a 1% agarose gel. The ethidium bromide-stained agarose gel is shown. Sizes of the DNA markers are indicated on the left.

RESULTS

Expression of RXR transcripts in NT2 cells

The expression of RXR isoforms in NT2 cells was studied using RT-PCR analysis. Total RNA was prepared from untreated or RA-treated (day 14) NT2 cells, and specific primers that amplified different isoforms (α, β, and γ) of human RXRs were used for the analysis (Table 1). Amplified products were cloned into pBluescript KS$^+$, and DNA sequencing was performed to confirm their identities. All isoforms of RXRs (α, β, and γ) were found to be expressed in both untreated and RA-treated NT2 cells (Fig. 1), and the expression was not affected after 14 days of RA treatment. A detailed time course study was performed to examine the expression of RXRα transcript during the process of RA-induced neuronal differentiation. Specific primers flanking the full-length cDNA of the human RXRα were employed for the RT-PCR analysis (Table 1). Human cyclophilin gene was used as an internal control for the quantity of total RNA as previously described (Cheung et al., 1996) (Fig. 2). Southern blot analysis using specific human RXRα cDNA probes revealed that the expression of RXRα transcript was up-regulated along the

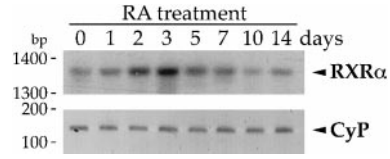

FIG. 2. Expression of RXRα transcript during the RA-induced neuronal differentiation of NT2 cells. NT2 cells were treated with 10 μ*M* *t*-RA for 0–14 days as indicated. Total RNA was prepared, and RT-PCR (25 cycles) was performed using specific primers (see Table 1) amplifying the full length (RXRα). **Upper panel:** Results of the subsequent Southern blot analysis performed using specific human RXRα cDNA probes generated by the partial RXRα primers are shown. **Lower panel:** The ethidium bromide-stained gel of the amplified partial cDNA fragment of the human cyclophilin gene (CyP) to indicate the equal amount of total RNA used for the analysis is shown. Sizes of the DNA markers are indicated on the left.

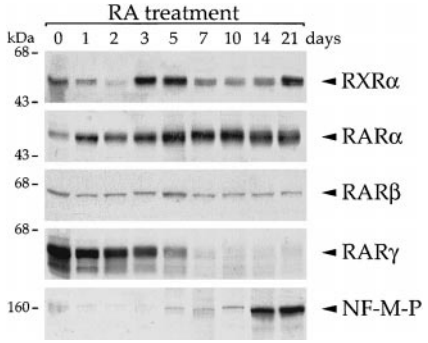

FIG. 3. Expression of RXRα, RARα, RARβ, and RARγ during the neuronal differentiation of NT2 cells induced by *t*-RA treatment. NT2 cells were treated with *t*-RA (10 μ*M*) for 1–21 days as indicated. Total proteins were prepared and western blot analysis was performed using antibodies specific for RXRα and different RARs (RARα and RARγ) as indicated on the right. NF-M-P was used as a control neuronal marker. Positions of the protein size markers are as indicated on the left.

course of *t*-RA treatment. The expression level of RXRα peaked at day 3 and then was reduced to its basal level at day 10 (Fig. 2). Similar results were obtained by reducing the number of PCR cycles (data not shown).

Expression of RXRα and RARs following treatment with *t*-RA and 9*c*-RA in NT2 cells

Western blot analysis of the expression of RXRα protein during the RA-induced neuronal differentiation of NT2 cells revealed a biphasic up-regulation of RXRα protein expression (Fig. 3). The expression of RXRα was up-regulated following treatment with 10 μ*M t*-RA for 3 days; the expression was subsequently reduced and a high level of RXRα expression was observed again at day 21 (Fig. 3). As RXRα could form heterodimers with either RARα or RARγ (Chiba et al., 1997), the expression of RARα and RARγ was also examined. Unlike the expression profile of RXRα, the expression of RARα was rapidly increased after 1 day of *t*-RA (10 μ*M*) treatment and was maintained throughout the 21-day period (Fig. 3). On the other hand, the expression of RARβ was relatively constant during the course of RA treatment, whereas RARγ was down-regulated by RA, especially after 5–7 days of *t*-RA treatment (Fig. 3). Thus, distinct expression profiles of RXRα, RARα, and RARγ were observed during the RA-induced neuronal differentiation of NT2 cells. Similar results were obtained by reducing the final concentration of *t*-RA to 2 μ*M* (data not shown).

Similar to *t*-RA, treatment of NT2 cells with 9*c*-RA was previously reported to induce neuronal phenotypes in NT2 cells (Kurie et al., 1993). To examine whether the expression of RXRα and RARs was also regulated by 9*c*-RA, NT2 cells were treated with 2 μ*M* 9*c*-RA for 1–21 days and western blot analysis was performed. We found the expression profiles of RXRα, RARα, RARβ, and RARγ observed in NT2 cells upon treatment with 2 μ*M* 9*c*-RA were similar to those obtained using *t*-RA (Fig. 4). That is, the expression of RARα was up-regulated, RARβ was not significantly affected, and RARγ expression was down-regulated along the course of 9*c*-RA treatment. Up-regulation of RXRα expression was observed when the NT2 cells were treated with 9*c*-RA for >5 days (Fig. 4). Thus, the changes in the expression profiles of RXR and RARs were independent of the form of RA used to induce the process of neuronal differentiation.

Binding of RXRα to RXRE

To examine the binding activities of RXRα, we performed the gel retardation assays using radioactively labeled DNA probes to examine the interaction of RXRα with the RXRE. Band shift was observed when the RXRE oligonucleotides were incubated with nuclear extracts obtained from the RA-treated NT2 cells (Fig. 5). The intensity of the DNA–protein complex increased with increased amount of nuclear extracts added (Fig. 5A). The binding was reduced by specific polyclonal antibody that detected the RXRα proteins (Fig. 5B), demonstrating that the DNA–protein complex contained RXRα. The specificity of the DNA–protein binding was also confirmed by the addition of excess unlabeled RXRE probes, which could prevent the binding (Fig. 5C). Furthermore, the addition of nonspecific competitors such as NS, AP2, and SP1 could not compete with the RXRE in its binding to RXRα. These data show that the induced RXRα is able to bind specifically to the RXRE, which will trigger the subsequent signaling cascade in RA action. It is noteworthy that the addition of excess unlabeled RARE probes could slightly reduce the binding (data not shown), raising the possibility that the protein complexes that bound to the RXREs might also contain the RXR/RAR heterodimers.

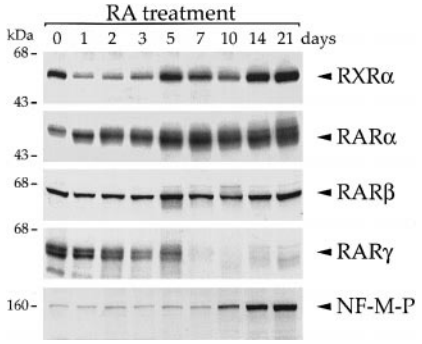

FIG. 4. Expression of RXRα, RARα, RARβ, and RARγ during the neuronal differentiation of NT2 cells induced by 9*c*-RA treatment. NT2 cells were treated with 9*c*-RA (2 μ*M*) for 1–21 days as indicated. Total proteins were prepared and western blot analysis was performed using antibodies specific for RXRα and different RARs (RARα and RARγ) as indicated on the right. NF-M-P was used as a control neuronal marker. Positions of the protein size markers are as indicated on the left.

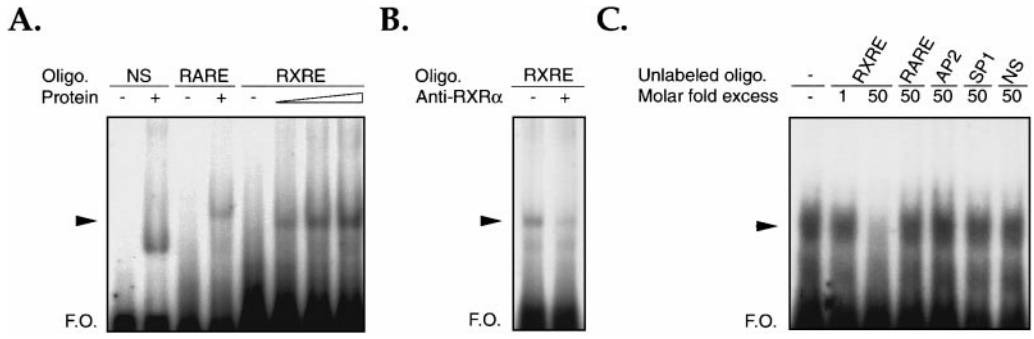

FIG. 5. Gel retardation assays demonstrate the binding of RXRα to the RXRE. **A:** Nuclear extracts from 4×10^5 NT2 cells (+) were mixed with radioactively labeled oligonucleotides NS and RARE (lanes 2 and 4). Nuclear extracts equivalent to different numbers of NT2 cells (2×10^5, 4×10^5, and 6×10^5) were mixed with radioactively labeled RXRE. No nuclear extract (−) was added in the negative control reactions. **B:** Nuclear extracts from 4×10^5 NT2 cells were mixed with radioactively labeled RXRE. Where indicated (+), the specific polyclonal anti-RXRα antibodies were added. **C:** Similar gel retardation assays were performed by mixing nuclear extracts (4×10^5 NT2 cells) with radioactively labeled RXRE. As indicated, a 1- or 50-fold molar excess of unlabeled oligonucleoides (RXRE, RARE, AP2, SP1, and NS) was added. Incubation mixtures were resolved in a 5% polyacrylamide gel in 0.5× Tris/borate/EDTA. The specific RXRE-RXRα complexes (arrowheads) and the free radioactively labeled oligonucleotides (F.O.) are indicated.

Subcellular localization of RXRα in NT2 neurons

Possible NT2 cell types that might express RXRα include the committed CNS precursor cells that are ready to acquire their neuronal phenotypes as well as the terminally differentiated CNS neurons resulting from the prolonged RA treatment. The protein expression of RXRα was examined in enriched NT2 neurons. It was demonstrated that enriched NT2 neurons expressed a high level of RXRα protein (Fig. 6A). Furthermore, immunofluorescent staining using specific RXRα antibodies demonstrated the expression of RXRα in both the somatic region and the neurites of the NT2 neurons (Fig. 6B).

To further elucidate the possible roles of RXRα, we have examined the subcellular localization of RXRα in the NT2 neurons. Double-staining with antibodies specific for RXRα and the dendritic marker (MAP2) revealed the co-localization of MAP2 and RXRα at the somatic region of the NT2 neurons (Fig. 7A). Furthermore, the expression of RXRα appeared to be associated with the axonal functions of the CNS neurons. Double-staining of the NT2 neurons with TH- and VIP-specific antibodies revealed the co-localization of TH or VIP immunoreactivity with RXRα in the cell bodies and neurites of the NT2 neurons (Fig. 7B and C, respectively). This finding demonstrated that the expression of RXRα could be observed in different subpopulations of human CNS neurons.

DISCUSSION

We report here the first study on the expression and regulation of RXRα during the prolonged treatment of NT2 cells with RA. The biphasic up-regulation of RXRα observed during the RA-induced neuronal differentiation of NT2 cells is independent of the type of RA used. RXRα can bind specifically to the RXRE. Furthermore, distinct expression profiles of RXRα, RARα, and RARγ were observed during the RA-induced neuronal differentiation of NT2 cells. Our finding on the regulatory profiles of RXRα and RARs is consistent with the previous suggestion that RXRs can exert their specific and redundant functions by forming heterodimers with different RARs (Chiba et al., 1997).

Recent observations suggest that RXRα might play essential roles in neuronal differentiation as well as functioning of the CNS neurons. For example, the expression of RXRα was detected in the neural tubes of chicken embryos (Hoover and Glover, 1998). Moreover, a recent report demonstrated the restrictive expression of RXRα in developing rat brains, such as the dentate gyrus and cranial nerve nuclei (Zetterström et al., 1999). To mediate different physiological functions, RXRα can form either homodimers or heterodimers with the RARs. Previous studies have reported that the RXRα/RARα heterodimers mediated the endodermal differentiation of the F9 EC cells (Chiba et al., 1997) and that RXRα might be involved in mediating the growth arrest and differentiation of human neuroblastoma cells by forming heterodimers with different RARs (Giannini et al., 1997). In the present study, we have demonstrated that both *t*-RA and the 9*c*-RA could regulate the expression of RXRα in a similar manner. As the formation of RXR homodimers could be induced only by 9*c*-RA (Heyman et al., 1992; Allenby et al., 1993), our finding is consistent with the possibility that RXRα might form heterodimers with RARα to mediate the specific neuronal functions in NT2 cells. Preliminary studies in our laboratory have demonstrated the co-localization of RXRα and RARα in the terminally differentiated NT2 neurons (data not shown).

The expression of RXRα in the cell bodies and neurites of enriched NT2 neurons, as reported in this study, suggests that RXRα might also mediate the cellular

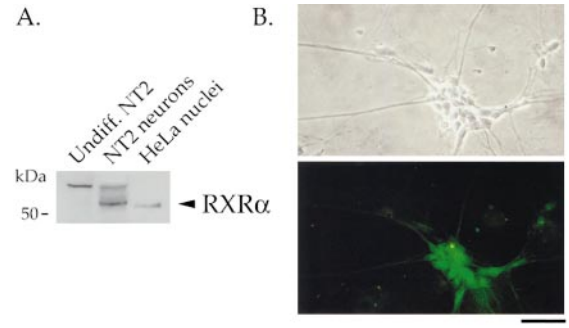

FIG. 6. Expression of RXRα in enriched NT2 neurons. **A:** Western blot analysis of the expression of RXRα in total proteins prepared from undifferentiated NT2 cells (Undiff. NT2) and NT2 neurons as well as HeLa nuclei, which were used as a positive control. **B:** Immunocytochemical analysis of the expression of RXRα in the NT2 neurons. **Upper panel:** Phase contrast micrograph; **lower panel:** enriched NT2 neurons stained with polyclonal rabbit anti-RXRα antibodies. Bar = 2.5 μm.

functions of the CNS neurons. Furthermore, double-staining of the NT2 neurons demonstrated the co-localization of MAP2 and RXRα at the somatic regions of the NT2 neurons. As MAP2 is a cytoskeletal protein localized at the dendrites (Garner et al., 1988; Morales and Fifkova, 1989), our findings suggested the association of RXRα with the somatoaxonal functions of the CNS neurons. It is noteworthy to observe that there is also a strong association between the RXRα immunoreactivity and the TH or VIP immunoreactivities. Because they represent two different neuronal populations in the CNS, namely, dopaminergic and GABAergic (Csillik et al., 1998), RXRα might potentially play a role in the neuronal functions of both the excitatory and the inhibitory CNS neurons.

The expression of RARγ during RA-induced neuronal differentiation was also examined in this study. This receptor component has previously been suggested to be important in mediating the RA-induced neuronal differentiation when the expression of RARγ was found to be absent in RA-resistant NT2 cells (Moasser et al., 1994). Although these RA-resistant NT2 cells resume their abilities to differentiate when transfected with RARγ, they do not differentiate into neurons but become mesenchymal cells (Moasser et al., 1995). Our findings on the reduced expression of RARγ at the later stages of neuronal differentiation suggest that the terminal differentiation of NT2 cells might require the participation of receptor molecules other than RARγ.

In summary, our studies provide the first demonstration of the expression profile of RXRα, RARα, RARβ, and RARγ following prolonged treatment with RA. Taken together with the finding of the ability of induced RXRα to bind specifically to RXRE and the demonstration of RXRα expression in the somatoaxonal regions of the NT2 neurons, our observations suggest that RXRα might play an important role in mediating the RA signaling and in the cellular functions of populations of human CNS neurons.

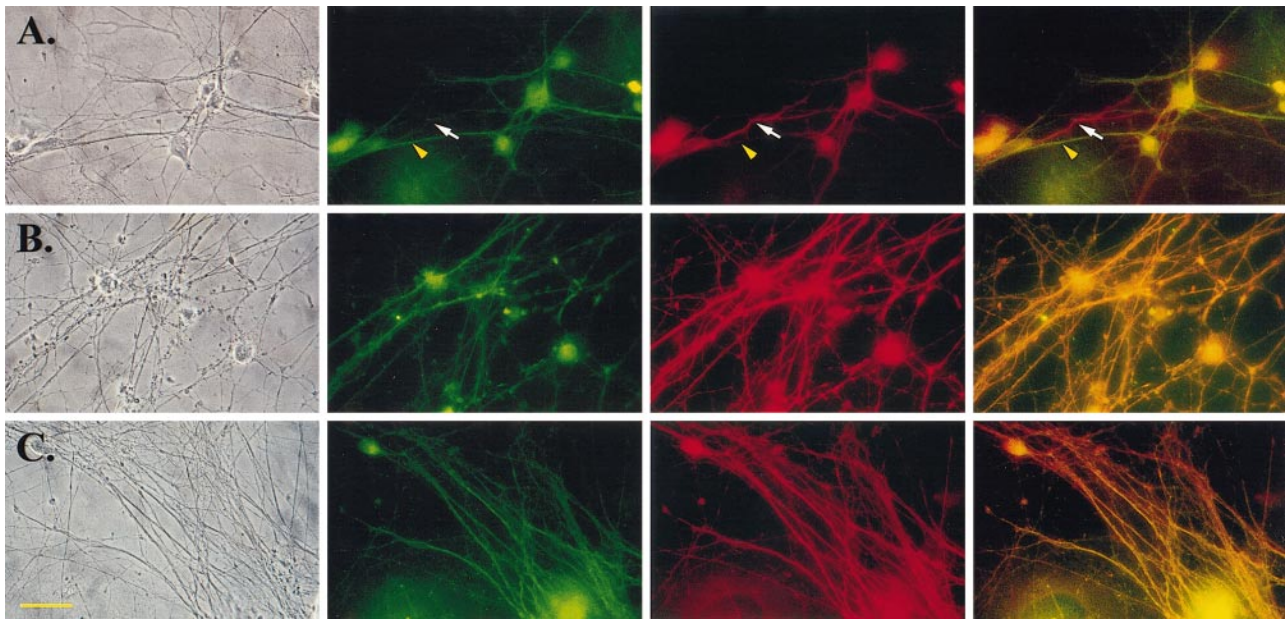

FIG. 7. Subcellular localization of RXRα in NT2 neurons. Immunocytochemical analysis was performed by double-staining of the NT2 neurons with specific antibodies against RXRα and MAP2 (**A**), TH (**B**), and VIP (**C**). Results of the phase contrast micrograph are indicated in the first column on the left, RXRα in the second column, and the specific neuronal markers in the third column. Results obtained by superimposing the second and third columns are indicated in the fourth column on the right. The $RXR\alpha^+/MAP2^-$ axon and the $RXR\alpha^-/MAP2^+$ dendrite are indicated by the yellow arrowhead and white arrow in A, respectively. Bar = 2.5 μm.

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