An Autocrine Loop Involving Ret and Glial Cell–Derived Neurotrophic Factor Mediates Retinoic Acid–Induced Neuroblastoma Cell Differentiation

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Abstract

In several neuroblastoma cell lines, retinoic acid (RA)–induced differentiation is coupled to increased expression of functional neurotrophic factor receptors, including Trk family receptors and the glial cell–derived neurotrophic factor receptor, Ret. In several cases, increased expression is dependent on signaling through TrkB. Unlike TrkA and TrkB, Ret has never been implicated as a prognostic marker for neuroblastomas. SK-N-BE(2) cells do not express any of Trk family receptors; therefore, they are a choice system to study the specific role of Ret in RA-induced differentiation. Using a 2′-fluoro-RNA aptamer and a truncated Ret protein as specific inhibitors of Ret, we show that RA-induced differentiation is mediated by a positive autocrine loop that sustains Ret downstream signaling and depends on glial cell–derived neurotrophic factor expression and release. This report shows that in SK-N-BE(2) cells, stimulation of Ret is a major upstream mechanism needed to mediate RA-induced differentiation. These results provide important insights on the molecular mechanism of RA action, which might be relevant for the development of biologically based therapeutic strategies.
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Introduction

Neuroblastomas are childhood tumors of the sympathetic nervous system, the majority of which are aggressive metastatic tumors with poor clinical outcome (1). Expression of the TrkA receptor has been identified as prognostic for favorable neuroblastoma, whereas TrkB and its ligand, the brain-derived neurotrophic factor, are frequently expressed in unfavorable aggressive tumors. Although the receptor for the glial cell–derived neurotrophic factor (GDNF), Ret, is expressed in most neuroblastomas, unlike TrkA and TrkB, it has not been implicated as a prognostic marker for neuroblastoma. Retinoic acid (RA) is a naturally occurring compound related to vitamin A that induces several human neuroblastoma cells to differentiate *in vitro* toward a neuronal-like phenotype characterized by neurite outgrowth, changes in the distribution of neurofilaments, and organization of these neuron-like cells into ganglion-like clusters (2, 3). RA derivatives are being employed in the therapy of neuroblastoma, and patients treated with RA have increased survival rates (4-6). Although little is known about the specific pathways that mediate RA action, in most cases, the expression and function of the TrkB receptor is required. Furthermore, a variety of polypeptide growth factors, including nerve growth factor, the brain-derived neurotrophic factor, and GDNF family ligands, have been shown to induce differentiation and/or apoptosis of most neuroblastoma cells *in vitro* (for a review see, refs. 1, 3, 7). How each of these processes contributes to the phenotype induced by RA remains an issue of fundamental importance.

The GDNF family ligands are characterized as potent survival factors for midbrain dopaminergic neurons as they support *in vivo* the survival of many types of neuronal cells, in both central and peripheral nervous systems, including sympathetic, parasympathetic, sensory, and enteric neurons (8). Their biological actions are mediated by a multicomponent receptor complex that contains a common signaling component, the Ret receptor, and one of the four members of the GFRα family (GFRα1-α4) coreceptors (9).

Ret is a receptor tyrosine kinase that is expressed in several cell lineages, including sympathetic neurons and adrenal chromaffin cells, from which neuroblastoma originates. Signaling through Ret is necessary for the differentiation of neuronal precursor cells to mature autonomic neurons. Indeed, mutations that affect the Ret signaling pathways cause Hirschsprung's disease in humans. Furthermore, mice that lack GDNF, GFRα1, or Ret share a phenotype characterized by kidney agenesis and absence of many parasympathetic and enteric neurons (7).

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As mentioned above, although Ret has been consistently found expressed in neuroblastoma tumor specimens and in most neuroblastoma-derived cell lines, no clear evidence has been reported that may support its involvement in neuroblastoma pathogenesis, and its expression does not correlate with disease aggressiveness (10, 11). On the other hand, in primary cultures, GDNF and neurturin cooperate with RA to induce neurite outgrowth and GDNF induces neuronal differentiation in low-grade but not in high-grade neuroblastoma cells (12, 13). Treatment of several human neuroblastoma cell lines with RA rapidly induces Ret expression by several times. Increased Ret expression is an early event followed by the expression of a differentiated phenotype, which includes expression of growth-associated protein-43 (GAP-43), nerve growth factor-inducible protein (VGF), neurite outgrowth, and inhibition of cell proliferation (12, 14, 15).

All these evidences contribute to suggest that up-regulation of Ret expression could be important in determining the phenotype induced by RA treatment. In this study, we sought to better understand how activation of Ret participates to the differentiation of human neuroblastoma cells. To minimize the contribution of TrkB, here, we chose to use the human SK-N-BE(2) neuroblastoma cell line (hereafter named SK-N-BE), in which RA-induced differentiation is preceded by a rapid accumulation of Ret but not of Trk family receptors that are not or poorly expressed (14, 16). We used two specific interfering molecules to inhibit Ret function: the EC-Ret peptide that blocks Ret stimulation by competing for binding to the ligand-GFRα complex (17, 18) and the D4 aptamer that inhibits Ret activity by directly binding the receptor (19).

Our results show that in SK-N-BE cells RA-induced differentiation is preceded by the accumulation of two components of the transducing machinery, Ret and GDNF, whereas the levels of GFRα expression remain rather constant. Furthermore, stimulation of Ret is crucial to mediate the effects of RA, thus overcoming the malignant phenotype. Most importantly, hampering Ret activity abrogates RA-induced differentiation, which indicates that RA induces differentiation of SK-N-BE cells by modulating the expression of the Ret transducing machinery.

Results

RA-Dependent Ret and Extracellular Signal-Regulated Kinase Activation Involves Ret Dimerization

In SK-N-BE cells, the low basal level of the Ret protein was rapidly induced by RA in a time- and dose-dependent manner (Fig. 1A, *left* and *middle*, respectively) and levels remained unchanged up to 72 hours of RA treatment (data not shown). Increase in the level of the protein was accompanied by stimulation of its activity as assessed by increased levels of tyrosine-phosphorylated Ret (Fig. 1A, *right*).

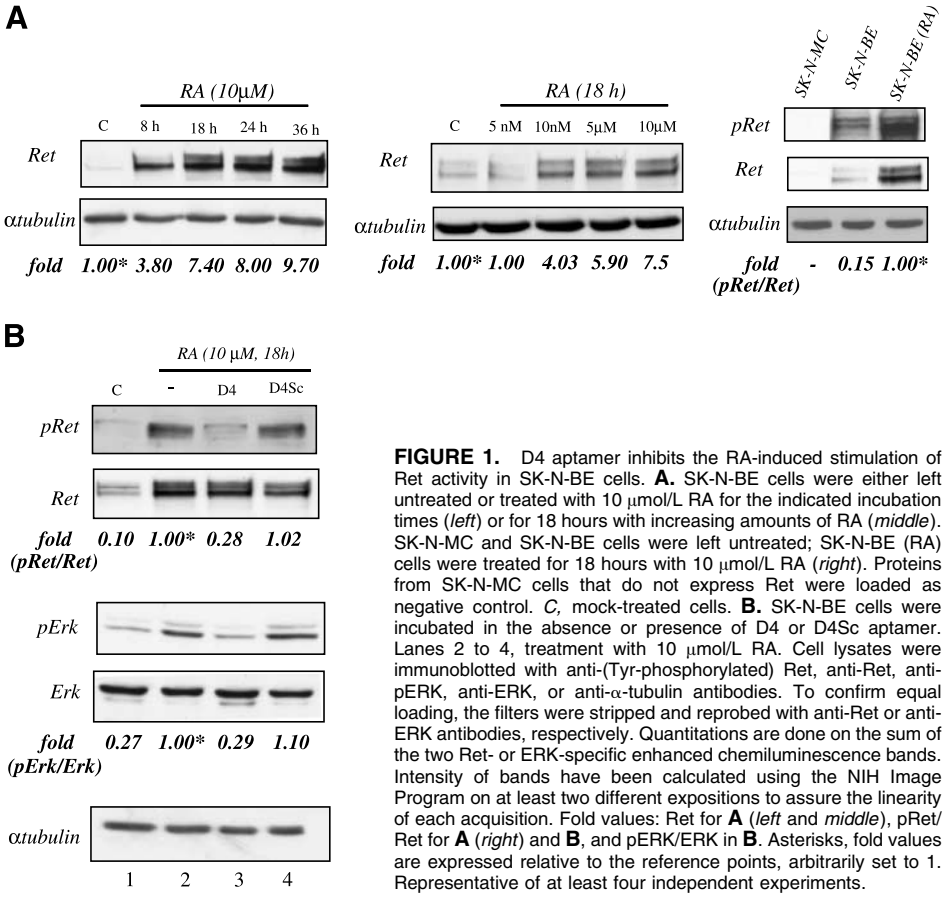

FIGURE 1. D4 aptamer inhibits the RA-induced stimulation of Ret activity in SK-N-BE cells. **A.** SK-N-BE cells were either left untreated or treated with 10 μmol/L RA for the indicated incubation times (*left*) or for 18 hours with increasing amounts of RA (*middle*). SK-N-MC and SK-N-BE cells were left untreated; SK-N-BE (RA) cells were treated for 18 hours with 10 μmol/L RA (*right*). Proteins from SK-N-MC cells that do not express Ret were loaded as negative control. *C,* mock-treated cells. **B.** SK-N-BE cells were incubated in the absence or presence of D4 or D4Sc aptamer. Lanes 2 to 4, treatment with 10 μmol/L RA. Cell lysates were immunoblotted with anti-(Tyr-phosphorylated) Ret, anti-Ret, anti-pERK, anti-ERK, or anti-α-tubulin antibodies. To confirm equal loading, the filters were stripped and reprobed with anti-Ret or anti-ERK antibodies, respectively. Quantitations are done on the sum of the two Ret- or ERK-specific enhanced chemiluminescence bands. Intensity of bands have been calculated using the NIH Image Program on at least two different expositions to assure the linearity of each acquisition. Fold values: Ret for **A** (*left* and *middle*), pRet/Ret for **A** (*right*) and **B**, and pERK/ERK in **B**. Asterisks, fold values are expressed relative to the reference points, arbitrarily set to 1. Representative of at least four independent experiments.

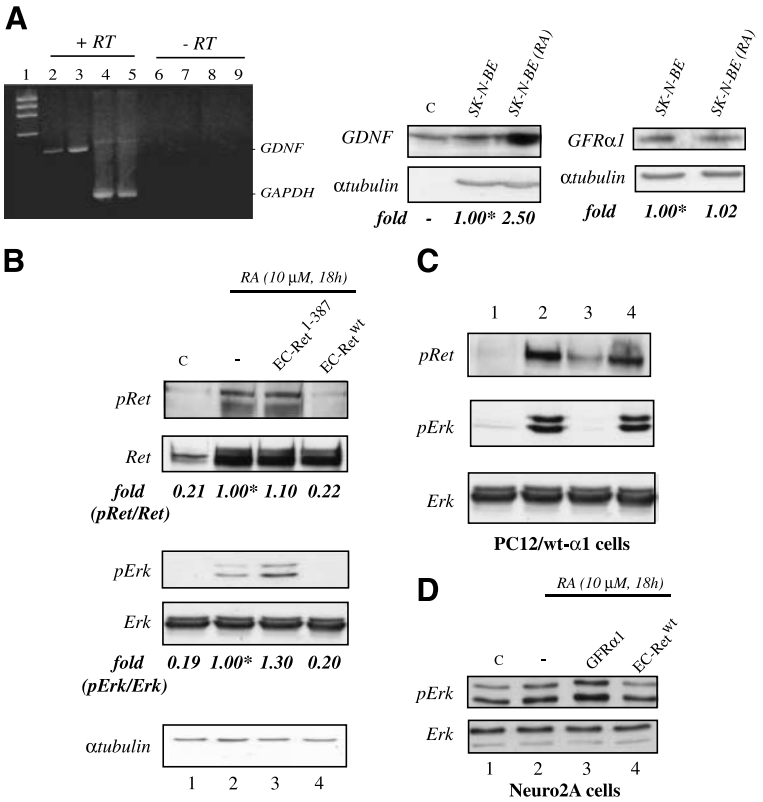

FIGURE 2. RA-dependent Ret activation is mediated by GDNF. **A.** Left, RNA isolated from SK-N-BE either left untreated or treated for 18 hours with 10 μmol/L RA was reverse transcribed and amplified as described in Materials and Methods. Samples loaded on the gel are as follows: lane 1, markers IX (Roche); lanes 2 and 3, amplification of GDNF; lanes 4 and 5, amplification of glyceraldehyde-3-phosphate dehydrogenase; lanes 3 and 5, cells were treated with RA; lanes 6 to 9, RNA samples as in lanes 2 to 5 but in the absence of RT as negative controls. Lysates from SK-N-BE or SK-N-BE (RA) cells were immunoblotted with anti-GDNF (*middle*) or anti-GFRα1 (*right*) antibodies. To confirm equal loading, the filters were hybridized with anti-α-tubulin antibodies. Middle, C, 40 ng purified GDNF used as a control of protein migration. **B.** SK-N-BE cells were incubated in the absence or presence of EC-Ret^{wt} or EC-Ret$^{1-387}$. Lanes 2 to 4, treatment with 10 μmol/L RA. Cell lysates were immunoblotted with anti-(Tyr-phosphorylated) Ret, anti-Ret, anti-pERK, anti-ERK, or anti-α tubulin antibodies. Quantitation and relative abundances are expressed relative to controls, arbitrarily set to 1 (see Fig. 1 legend). Representative of at least four independent experiments. **C.** PC12/wt-α1 cells were serum starved for 4 hours and then treated for 10 minutes with mock-treated cells (*lane 1*), 100 ng/mL GDNF (*lane 2*), or conditioned medium harvested from SK-N-BE cells treated with 10 μmol/L RA for 10 minutes (*lane 3*) or 18 hours (*lane 4*). Cell lysates were immunoblotted with anti-(Tyr-phosphorylated) Ret and anti-pERK antibodies. To confirm equal loading, the same filter was stripped and reprobed with anti-ERK antibodies. **D.** Neuro2A cells were incubated in the absence or presence of 1.6 nmol/L GFRα1 or EC-Ret^{wt}. Lanes 2 to 4, treatment with 10 μmol/L RA. Cell lysates were immunoblotted with anti-pERK or anti-ERK.

First, we asked whether RA-dependent Ret activation depends on the presence of an autocrine loop involving ligand-dependent dimerization or, rather, the receptor itself is transactivated by intracellular mechanisms acting on its kinase domain (20). To this end, we used a 2′-fluoro-RNA-based aptamer, named D4, which is a specific inhibitor of dimerization-dependent Ret activation but has no effects on Ret activation induced by mutations of the intracellular tyrosine kinase domain (19). As shown in Fig. 1B, treating cells with the D4 aptamer but not with an inactive control of scrambled sequence (D4Sc) drastically inhibited RA-dependent stimulation of Ret autophosphorylation that was reduced to basal levels (compare *lane 3* with *lane 1*). D4 has a specific effect on Ret activation but not on the RA-dependent induction of Ret expression, thus reducing the pRet/Ret ratio close to basal levels in the absence of RA (Fig. 1B).

As extracellular signal-regulated kinase (ERK) is a crucial downstream effector of Ret (21), we assessed the effect of RA treatment and D4 inhibition on ERK phosphorylation. Following RA treatment, the levels of ERK phosphorylation were highly increased (~4-fold increase) and persistent (Fig. 1B, compare *lane 2* with *lane 1*); treatment with the D4 aptamer resulted in a strong inhibition of the RA-induced phosphorylation of ERK (*lane 3*) that well mirrors the inhibitory effects of D4 on Ret activity. Taken together, results indicate that in SK-N-BE cells the activation of the ERK pathway is mediated mainly by the stimulation of Ret.

RA-Induced Stimulation of Ret and ERK Are Mediated by GDNF

These results suggest that RA-induced Ret activation is dependent on GDNF signaling. One possible implication is that GDNF and/or the GFRα1 coreceptor are as well expressed or induced, thus providing the three-component transducing complex. As shown in Fig. 2A, both GDNF mRNA (*left*) and protein (*middle*) are expressed in this cell line as detected by reverse transcription-PCR and Western blotting, respectively. Interestingly, however, a marked increase in protein levels was observed following RA treatment. In contrast, the GFRα1 protein level remained unchanged on RA treatment (Fig. 2A, *right*).

Because the simultaneous presence of all components of the Ret transducing complex supports the notion that Ret is stimulated by an autocrine loop sustained by GDNF, we wondered whether Ret activation depends on the synthesis and the extracellular secretion of GDNF. To this aim, we used a specific competitive inhibitor of Ret activity consisting of the entire Ret wild-type extracellular domain in a soluble form, EC-Ret^{wt}, which in the presence of GFRα1 protein sequesters GDNF, thus reducing the available ligand for the activation of the membrane-bound Ret receptor (17, 18). As shown in Fig. 2B, RA stimulates Ret and ERK phosphorylation, and treating cells with EC-Ret^{wt} kept these levels close to basal (compare *lane 4* with *lane 1*), thus indicating that Ret activity is stimulated by soluble GDNF secreted in the medium. As a negative control, we used a recombinant fragment of Ret (EC-Ret$^{1-387}$) that is unable to bind to GDNF and GFRα1 (18).

Consistent with these results, the GDNF secreted in the medium can stimulate Ret in an unrelated cell system. As shown in Fig. 2C, conditioned medium harvested from SK-N-BE cells treated with RA stimulates Ret and ERK activity in a PC12-derived cell line that expresses both human Ret and GFRα1 (PC12/wt-α1 cells), whereas no stimulation was induced by control medium from SK-N-BE cells treated for 10 minutes with the same amount of RA. Furthermore, as shown in Fig. 2D, the same loop is induced by RA in an unrelated neuroblastoma cell line, Neuro2A (compare *lane 4* with *lanes 3* and *2*).

These results together indicate that RA treatment of SK-N-BE cells cause GDNF induction and secretion in the culture medium where it activates the membrane-bound Ret receptor.

Furthermore, because Ret is stimulated by an autocrine loop sustained by GDNF, we asked whether the exposure of SK-N-BE cells to exogenous GDNF could also stimulate the expression of cellular GDNF. We found that GDNF treatment of SK-N-BE cells for 4 days resulted in an increase in GDNF RNA and protein levels comparable with that obtained in RA-treated cells (data not shown), indicating that the activation of Ret by GDNF leads to the induction of GDNF expression.

Ret Activity Is Needed for RA-Induced Differentiation

Activation of Ret-dependent signaling has been shown to induce growth arrest and neuronal differentiation of several neuroblastoma cell lines (15, 22, 23). As RA strongly activates Ret, it seems reasonable to hypothesize that RA-induced differentiation of SK-N-BE cells implicates the stimulation of Ret activity as a necessary step toward cell differentiation. If true, inhibiting Ret activity should also hamper the process of differentiation induced by RA. We first monitored the levels of two proteins whose expression is up-regulated on RA-induced differentiation in several neuroblastoma cell lines induced to differentiate, VGF and GAP-43 (24, 25). As expected, on 72 hours of RA treatment, the levels of both VGF and GAP-43 proteins were increased of ~4 and 20 times (Fig. 3A, *lane 2, top* and *middle*, respectively). Remarkably, inhibiting Ret by treating cells with the D4 aptamer kept both protein levels close to basal (compare *lane 3* with *lane 1*), whereas the D4sc had no effect (*lane 4*). The antagonist effects of D4 were also determined by measuring inhibition of neurite outgrowth following RA treatment. Undifferentiated SK-N-BE cells are round shaped with few short processes (Fig. 3C). Treatment with 10 μmol/L RA for 3 days caused the cells to display a mature neuronal morphology with neurite extensions that occasionally connected the cells (Fig. 3D). Interestingly, treatment with the D4 aptamer, but not with the D4Sc control, significantly inhibited the percentage of neurite outgrowth induced by RA (Fig. 3B; for morphology, compare Fig. 3E with Fig. 3D and F), thus indicating that RA-induced differentiation of SK-N-BE requires Ret activity. Consistently, inhibition of Ret signaling by with EC-Ret^{wt} competitive inhibitor caused a drastic inhibition of the RA-dependent induction of VGF and GAP-43 proteins (Fig. 3I, *top* and *middle*, respectively) and of neurite outgrowth (compare Fig. 3G with Fig. 3D and H).

To further determine the role played by Ret as a mediator of RA action, we then addressed the expression levels of tissue transglutaminase. Indeed, both the expression and the activity of tissue transglutaminase are strongly enhanced by RA and have been shown to be necessary for RA-induced cell differentiation and ERK activation in neuroblastoma cell lines (26-28). Thus, we asked whether RA induces tissue transglutaminase expression via a Ret-independent mechanism or whether RA requires Ret activity to regulate tissue transglutaminase levels. Consistent with previous reports (27), RA induces rapid accumulation of tissue transglutaminase (Fig. 3L, *top*). Furthermore, we show that inhibiting Ret activity impairs the RA-dependent induction of tissue transglutaminase expression (Fig. 3L, *bottom*). This indicates that RA-dependent induction of tissue transglutaminase needs the reconstitution of a functional Ret/GDNF/GFRα1 complex.

RA Treatment of SK-N-BE Cells Interferes with GDNF Acute Stimulation

Because our results indicate that in the absence of neurotrophin receptors the action of RA is mediated by GDNF, we asked whether RA and GDNF cooperate in inducing cell differentiation. Indeed, the possibility for GDNF to enhance the effects of RA in neuroblastoma primary cells (12) or stable cell lines (29) is still debating. First, we compared the ability to induce neurite outgrowth in SK-N-BE cells treated by either RA alone, GDNF alone, or RA and GDNF combined. As shown in Fig. 4A to E, treating cells with GDNF alone (Fig. 4B) is sufficient to induce a comparable extent of neurite outgrowth as RA alone (Fig. 4C) with respect to untreated cells (Fig. 4A). As predicted by our results, the simultaneous addition of GDNF and RA does not significantly enhance the extent of neurite outgrowth (Fig. 4D). We asked whether chronic stimulation of Ret by cell-autonomous secretion of GDNF may interfere with subsequent response to exogenous acute stimulation. Therefore, we determined whether following RA induction Ret is still competent to respond to exogenous GDNF stimulation. As shown (Fig. 4F), following 18 hours of RA treatment, stimulation with GDNF for 10 minutes further enhanced the extent of Ret tyrosine phosphorylation (indicating that the GDNF response was not saturated) but had no relevant effects on the downstream ERK activation (compare *lane 2* with *lane 4*). In good agreement with our previous studies (21), a likely explanation for the inability of GDNF to enhance pERK levels relies on the presence of a negative feedback, acting on pERK, and induced by the chronic stimulation of the receptor that inhibits the transmission of acute Ret signaling to the nucleus. This was further confirmed by the inefficacy of exogenous GDNF to cooperate with RA in inducing neurite outgrowth (Fig. 4A-E).

Furthermore, we wondered whether D4 aptamer could inhibit GDNF-induced Ret phosphorylation in RA-treated cells. We found that D4 was effective to inhibit Ret phosphorylation when added to RA-treated SK-N-BE cells simultaneously with GDNF but not when cells were treated with the ligand before addition of the aptamer (Fig. 4G, compare *lane 6* with *lane 8*). This indicates that D4 is able to interfere with GDNF-induced Ret phosphorylation (even in the presence of a strong acute stimulus), but it is unable to reverse the phosphorylation of the active complex once formed.

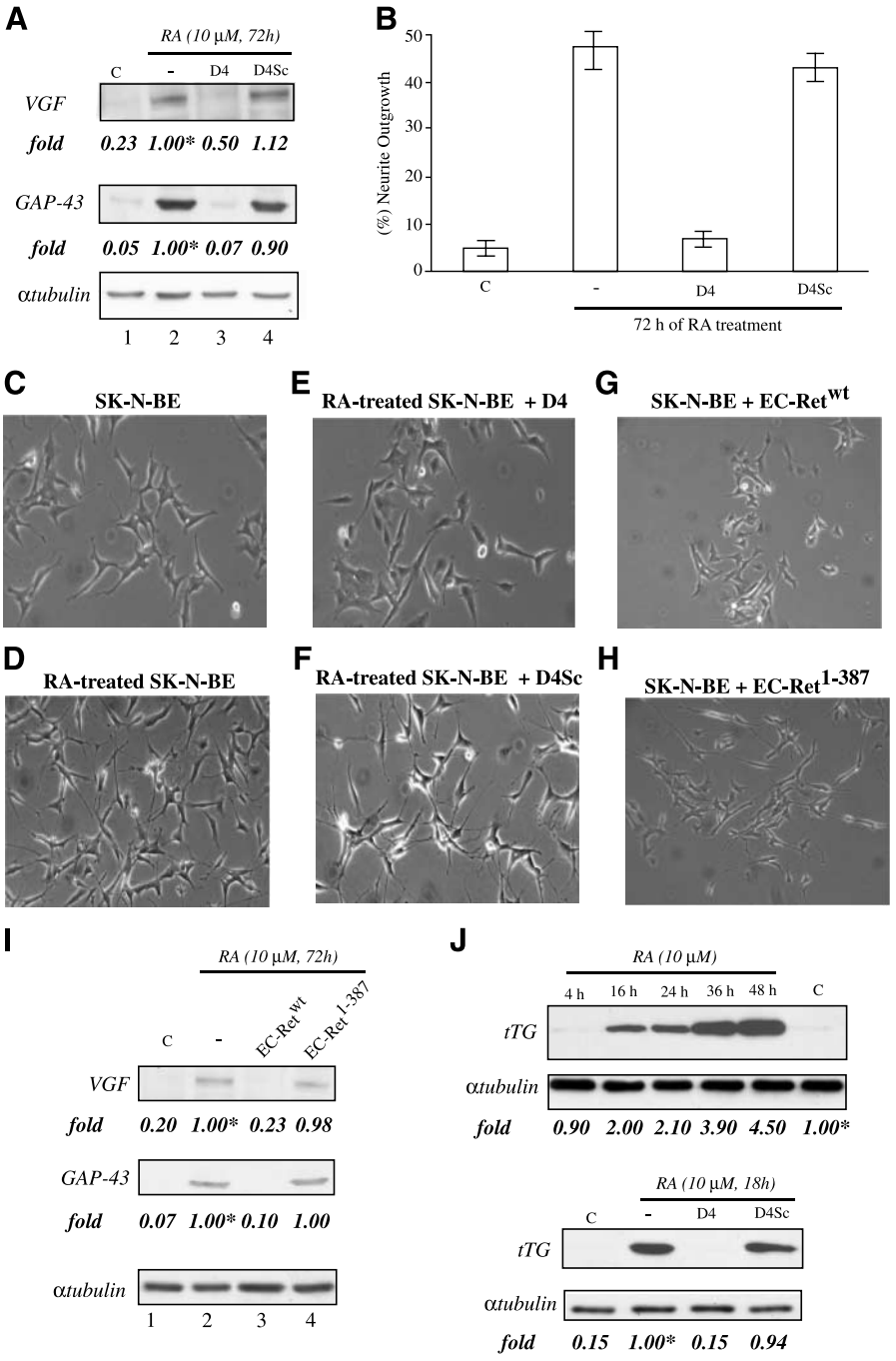

FIGURE 3. D4 aptamer inhibits RA-induced differentiation of SK-N-BE cells. SK-N-BE were left untreated or treated for 72 hours with RA either in the absence or presence of D4, D4Sc aptamer, EC-Ret^{wt}, or EC-Ret$^{1-387}$. **A.** Cell lysates were immunoblotted with anti-VGF and anti-GAP-43 antibodies. Equal loading was confirmed by immunoblotting with anti-α-tubulin antibodies. **B** to **H.** Following RA-treatment, the percentage of neurite outgrowth was calculated and reported as histogram. Data are percentage of neurite-bearing cells/total cells analyzed. Columns, average of three independent experiments. A neurite was operationally defined as a process outgrowth that was more than twice as long as the diameter of cell body. Microphotographs of cells were done by using a phase-contrast light microscope. **I.** Lysates from SK-N-BE left untreated or treated for 72 hours with RA in the absence or presence of EC-Ret^{wt} or EC-Ret$^{1-387}$ were immunoblotted as in **A**. In **A** and **I**, quantitations were done as in Fig. 1 and relative abundances are expressed relative to controls, arbitrarily set to 1. Representative of at least three independent experiments. **J.** Top, SK-N-BE cells were either left untreated or treated with RA for the indicated incubation times; bottom, SK-N-BE were incubated in the absence or presence of D4 or D4Sc aptamer. Cell lysates were immunoblotted with anti–tissue transglutaminase (*tTG*) antibodies. To confirm equal loading, the same filter was stripped and reprobed with anti-α tubulin antibodies. Quantitation and relative abundances are expressed relative to controls, arbitrarily set to 1 (see Fig. 1 legend). Representative of at least three independent experiments.

Discussion

Here, we show that in the SK-N-BE cell line a large part of the biological effects of RA are mediated by an autocrine loop that stimulates the activity of the Ret tyrosine kinase. We show that signaling through Ret is an early event that is crucial for the RA-induced expression of tissue transglutaminase, stimulation of ERK activity, and morphologic differentiation. The choice of SK-N-BE as model system allowed us to elucidate Ret involvement in RA-induced differentiation in the absence of the neurotrophin receptors, TrkA and TrkB. Indeed, an alternative scenario exists, implicating the cooperative action of multiple transducing signals in human neuroblastoma cell lines expressing both Trk family receptors and Ret. In this case, the simultaneous presence of different trophic factor receptors could contribute to the final neuronal-like phenotype induced by RA.

RA treatment of SK-N-BE cells is followed by a rapid increase in the expression and activity of both Ret and tissue transglutaminase and in the stimulation of ERK. We first showed that blocking Ret activity with either the D4 aptamer or

EC-Ret^{wt} abrogated both RA-induced tissue transglutaminase expression and ERK activation, thus indicating that both enzymes are tightly regulated by Ret activity. Furthermore, inhibiting Ret also hampers the RA-induced expression of the neuronal-like phenotype as determined by the lack of changes in cell morphology and by the low levels of *GAP-43* and *VGF* genes. Therefore, these results show that in SK-N-BE cells Ret is needed to mediate the induction of cell differentiation by RA. Whether tissue transglutaminase, ERK, or other enzymatic activities are also needed for differentiation remains to be determined (see below). The implication of Ret activity as a necessary mediator for RA is consistent with the observation that induction of Ret is an early event preceding the establishment of the differentiated phenotype and that GDNF induces differentiation in cooperation with RA in primary human neuroblastomas (12).

At least two mechanisms can be invoked for the stimulation of Ret activity by RA: (*a*) in analogy with what shown for the brain-derived neurotrophic factor receptor TrkB (16), implicates the expression and secretion in the medium of the ligand, which maintains an autocrine loop; (*b*) based on a ligand-independent transactivation mechanism, implicates that Ret activation is

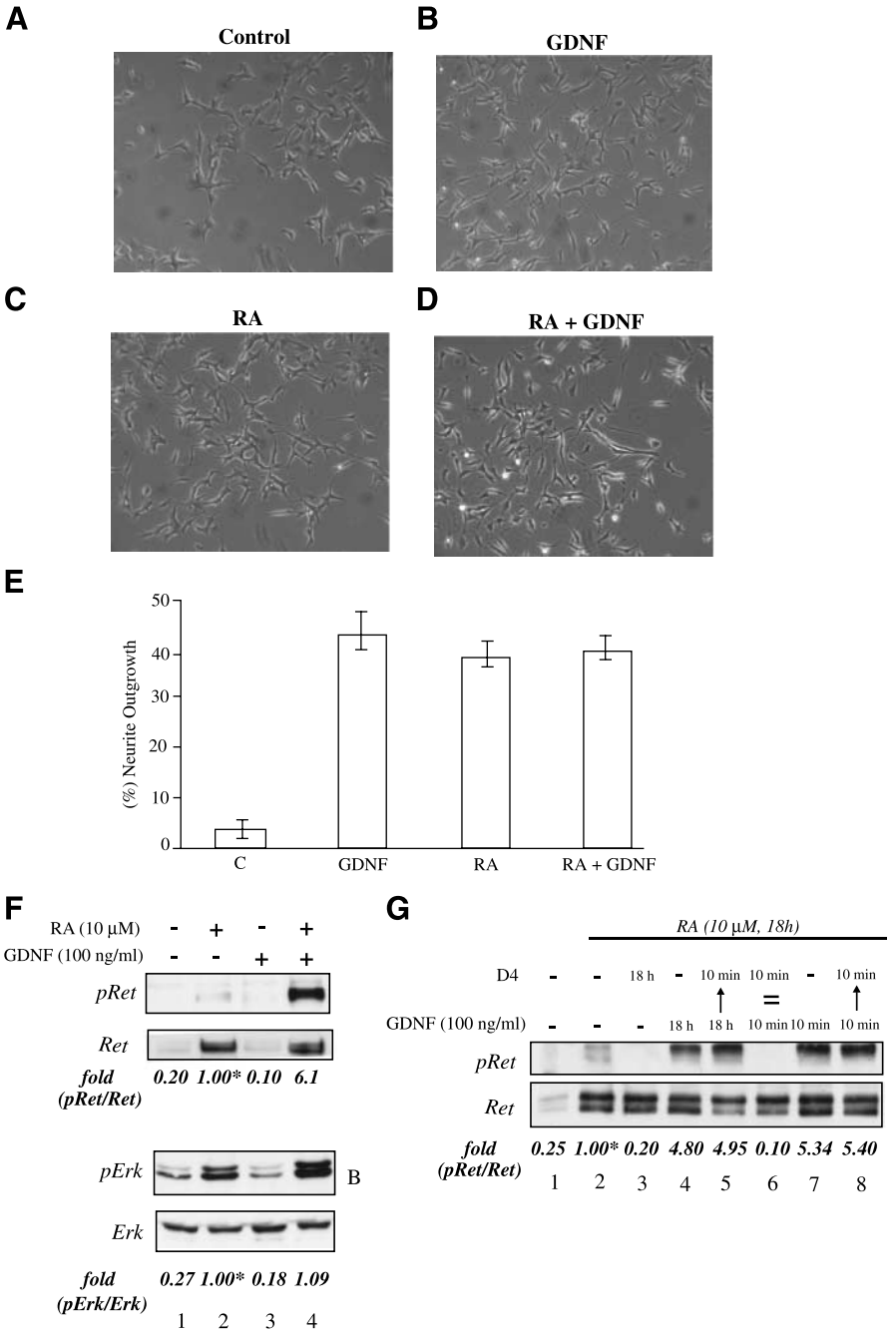

FIGURE 4. RA interferes with GDNF acute stimulation. SK-N-BE were left untreated or treated for 72 hours with GDNF (100 ng/mL), RA alone, or GDNF (100 ng/mL) + RA (**A-E**). Following RA treatment, the percentage of neurite outgrowth was calculated and reported as isogram as described in Fig. 3 legend. **F.** SK-N-BE cells were either left untreated or treated for 18 hours with RA, serum starved for 4 hours, and then treated for 10 minutes with GDNF. Cell lysates were immunoblotted with anti-(Tyr-phosphorylated) Ret, anti-Ret, anti-pERK, and anti-ERK antibodies. **G.** SK-N-BE cells were differently treated with RA, GDNF, and D4 aptamer (final concentration of 200 or 1,600 nmol/L for 10-minute or 18-hour incubation, respectively), and extracts were immunoblotted with anti-(Tyr-phosphorylated) Ret and anti-Ret antibodies. Samples loaded on the gel are from cells treated as follows: mock-treated cells (*lane 1*); cells treated for 18 hours with RA either in the absence (*lane 2*) or presence (*lane 3*) of D4; cells treated for 18 hours with RA + GDNF (*lane 4*); cells treated as in *lane 4* and then further incubated for 10 minutes with D4 (*lane 5*; *arrow,* incubation with GDNF precedes that with the D4); cells treated for 18 hours with RA and then incubated for 10 minutes with GDNF + D4 (*lane 6*; *double line,* GDNF and D4 have been added together on the cells); cells treated for 18 hours with RA and then incubated for 10 minutes with GDNF alone (*lane 7*); and cells treated for 18 hours with RA, stimulated for 10 minutes with GDNF, and then incubated for 10 minutes with D4 (*lane 8*; *arrow,* incubation with GDNF precedes that with the D4). In **A** and **B**, quantitation and relative abundances are expressed relative to controls, arbitrarily set to 1 (see Fig. 1 legend). Representative of at least three independent experiments.

mediated by intracellular molecular interactions (20). Therefore, the fact that the EC-Ret^{wt} peptide inhibits Ret activity and the resulting cell differentiation indicates that GDNF is secreted in the medium whereby it stimulates Ret by a cell-autonomous mechanism. Indeed, the EC-Ret^{wt} peptide competes for binding to GDNF, thus depleting the culture medium of the functional ligand. The assumption that in response to RA functional GDNF is secreted in the culture medium is further supported by the observation that the conditioned medium collected from RA-treated cells is sufficient to stimulate Ret activity in a heterologous cell system, thus indicating that the ligand secreted in the medium is still functional.

Tissue transglutaminase has been proposed to be implicated in a vast array of cellular processes, including the suppression of cell proliferation, differentiation, and signal transduction (30-34).

Compelling evidences indicate tissue transglutaminase activity as crucial to mediate RA-induced neuroblastoma cell differentiation. Following RA treatment, the levels of tissue transglutaminase increase in few days and its activity is necessary and sufficient to elicit neurite outgrowth likely involving activation of ERK and p38γ mitogen-activated protein kinase (27, 28, 35). Here, we show that in SK-N-BE cells inhibiting Ret activity, and thus differentiation, impairs stimulation of tissue transglutaminase whose levels remain basal even after RA treatment. As expected, RA also induces the persistent activation of ERK that in these cells is tightly regulated by Ret (36).[5] These results support a scenario in which RA promotes the expression of both Ret and its ligand GDNF, thus reconstituting a functional Ret/GDNF/GFRα1 complex This would promote several intracellular events that include persistent activation of ERK and accumulation of tissue transglutaminase, thus creating an alternative/supportive mechanism to brain-derived neurotrophic factor able to mediate the effects of RA.

In conclusion, these results show that stimulation of Ret tyrosine kinase is relevant to mediate the RA-induced differentiation. Indeed, RA stimulates both GDNF and Ret expression, thus inducing an autocrine loop that determines the final neuronal-like phenotype of these cells. This mechanism closely mimics the differentiation process of embryonal neuroblasts promoted by paracrine/autocrine stimulation of trophic factors acting on neuronal cells. Furthermore, given the relevance of the use of RA for therapeutic in neuroblastomas, the understanding of the molecular mechanisms that permit its action will reveal new strategies for combined approaches to combat tumor progression.

Materials and Methods

Cell Culture and Immunoblotting Analysis

Human SK-N-BE(2) neuroblastoma cells (American Type Culture Collection, Manassas, VA) and the mouse neuroblastoma cells, Neuro2A, were grown in DMEM supplemented with 2 mmol/L L-glutamine, 10% heat-inactivated fetal bovine serum (Hyclone, Celbio, South Logan, UT), and 50 μg/mL gentamicin. All-*trans* RA (Sigma, St. Louis, MO) was dissolved (10 mmol/L) in DMSO and diluted at the indicated concentration in serum-containing culture medium. Neuroblastoma cells (160,000 per 3.5 cm plate) were treated for 18 hours with 1 mL culture medium containing 10 μmol/L RA plus 50 μg of the indicated EC-Ret protein or plus 1600 nmol/L of the indicated aptamer after a short denaturation-renaturation step (19). To evaluate the effects of RNA aptamers and EC-Ret proteins on cell differentiation, cells were incubated in 12-well cell plate with 10 μmol/L RA together with 3 μmol/L aptamer or 20 μg/mL EC-Ret proteins. The concentration of the aptamer was calculated to ensure the continuous presence of a concentration of at least 200 nmol/L; this treatment was renewed every 24 hours, which takes into account the half-life of the D4 aptamer in 10% serum (~6 hours).[5] At 48 and 72 hours of RA treatment, 30 cells per frame were counted and scored as having neurites or not. At least 15 random fields were photographed with a phase-contrast light microscope. A neurite was operationally defined as a process outgrowth that was more than twice as long as the diameter of cell body. Growth conditions for PC12/wt-α1 cells were described previously (17).

Cell extracts and immunoblotting analysis were done as described (17). Antibodies used were anti-Ret (H-300), anti-VGF (R-15), anti-ERK1 (C-16), anti-GDNF (D-20), anti-GFRα1 (N-18; Santa Cruz Biotechnology, Inc., Santa Cruz CA); anti-(Tyr-phosphorylated) Ret, anti-phospho-p44/42 mitogen-activated protein kinase (E10; Cell Signaling, Beverly, MA); anti-GAP-43 (Zymed Laboratories, Inc., South San Francisco, CA); anti-α-tubulin (DM1A; Sigma); and anti–tissue transglutaminase antibodies (kindly provided by G. Peluso, CNR, Naples, Italy; ref. 37). Four independent experiments were done.

Ret Inhibitors

The entire Ret receptor extracellular portion (EC-Ret^{wt}) and the protein containing the first three NH_2-terminal CLDs of EC-Ret^{wt} but lacking CLD4 and CRD (named EC-Ret$^{1-387}$) were produced as reported previously (17, 18). D4 is a 93-base 2′-fluoro-RNA-based aptamer that specifically binds the extracellular domain of Ret thus interfering with ligand-induced stimulation of its intrinsic tyrosine kinase activity (19).

Reverse Transcription-PCR Analysis

Total RNA was isolated using a RNA extraction kit (Ambion, Austin, TX). RNA (5 μg) was reverse transcribed for 60 minutes at 42°C in 20 μL volume reaction mixture containing 20 units MMV-RT (Roche, Basel, Switzerland) and random hexanucleotides (Amersham Pharmacia, Uppsala, Sweden). The resulting cDNA fragments were used as PCR templates. The forward and reverse primers were *GDNF* 5′-TCAGTTCGATGATGTCATGG-3′ and 5′-CACACCTTT-TAGCGGAATGC-3′ and *glyceraldehyde-3-phosphate dehydrogenase* 5′-CATCAAGAAGGTGAAGC-3′ and 5′-TCTTAC-TCCTTGGAGGCCAT-3′.

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