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ORIGINAL ARTICLE

Retinoic acid receptors and tissue-transglutaminase mediate short-term effect of retinoic acid on migration and invasion of neuroblastoma SH-SY5Y cells

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Long-term treatment with all trans-retinoic acid (RA) induces neuronal differentiation and apoptosis. However, the effect of short-term RA treatment on cell proliferation, migration and invasion of neuroblastoma cell lines (SH-SY5Y and IMR-32) remains unclear. RA induces expression of tissue-transglutaminase (TGase) and promotes migration and invasion after 24 h of treatment in SH-SY5Y cells, but not in IMR-32 cells. RA receptor (RAR) agonist (4-(E-2-[5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl]-1-propenyl) benzoic acid) and RAR/retinoid X receptor (RXR) agonist (9-*cis*-RA) promote expression of TGase, migration and invasion of SH-SY5Y cells, while RXR agonist has no significant effect. RAR antagonist blocks RA effect on migration and invasion, indicating that RAR receptors are required. Retinoid receptors are expressed and activated by RA in both cell lines. However, only transient activation of RAR is observed in IMR-32 cells. These findings suggest that different responses observed in SH-SY5Y and IMR-32 cells could be due to differential activation of retinoid receptors. Overexpression of TGase has no effect on migration or invasion, while overexpression of antisense TGase blocks RA-induced migration and invasion, indicating that other molecules along with TGase mediate RA effects. In addition to the long-term effects of RA that are coupled with cell differentiation, short-term effects involve migration and invasion of neuroblastoma SH-SY5Y cells.
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Introduction

Neuroblastoma is the most common childhood malignancy arising due to improper differentiation of neuronal crest-derived cells of the adrenal medulla or sympathetic neurons, accounting for 8–10% of all childhood cancers (Castleberry, 1997; Cianfarani and Rossi, 1997). Although neuroblastoma is treatable when detected at an early stage, advanced-stage metastatic disease accounting for approximately 80% of patients is largely incurable (Frappaz *et al*., 2000; Schmidt *et al*., 2000; Westermann and Schwab, 2002). The causes of neuroblastoma and factors influencing the progression of the disease are not fully understood.

All *trans*-retinoic acid (RA; active metabolite of vitamin A) plays a prominent role in regulating the transition of proliferating precursor cells (such as embryonal carcinoma cells, hematopoietic and neuronal precursors) to postmitotic differentiated cells (Yao *et al*., 1995; Reynolds, 2000; Antonyak *et al*., 2001; Tucholski *et al*., 2001; Reynolds *et al*., 2003). RA functions by binding to two families of nuclear receptors: the RA receptors (RARs) family (RARα, β and γ), binding both RA and *9-cis* RA, and the retinoid X receptors (RXRs) family (RXRα, β and γ), preferentially binding *9-cis* RA (Heyman *et al*., 1992; Chambon, 1996; Maden, 2002). Though RA can bind only to the RARs, activated RAR heterodimerizes with RXR and RAR/RXR heterodimers bind to RA response element (RARE), resulting in transcriptional activation. Alteration in RAR expression or function has been observed in a variety of cancers; loss of RARβ, a probable tumor suppressor gene, is associated with different tumors including lung and squamous cell carcinoma, breast, stomach and prostrate cancers (Xu *et al*., 1994, 1997a, b; Qui *et al*., 1999; Lotan *et al*., 2000; Klaassen *et al*., 2001; Soprano and Soprano, 2002; Emionite *et al*., 2003; Niles, 2004).

Several studies have proposed retinoid-induced neuronal differentiation and/or apoptosis as the basis for tumor regression during chemotherapy, but little information is available on the relative contribution of individual retinoid receptors (Ponthan *et al*., 2001, 2003; Reynolds and Lemons, 2001). In SH-SY5Y cells, RA-induced differentiation to neuronal type requires 5–7 days. Previous studies have shown reduced invasion

capacity in SH-SY5Y cells after longer RA treatments, suggesting that invasive potential is a property of undifferentiated cells (Voigt and Zintl, 2003). However, the short-term effects of RA on migration and invasion of neuroblastoma cells are not known.

RA activates tissue-transglutaminase (TGase) and phosphatidylinositol 3-kinase (PI3K)-dependent pathways in SH-SY5Y cells after 24 h treatment, which are involved in migration and invasion of cancer cells (Meyer *et al*., 2001; Pola *et al*., 2003; Singh *et al*., 2003; Mehta *et al*., 2004; Pan *et al*., 2005). Hence, activation of these in undifferentiated neuroblastoma cells could positively influence their potential to migrate and invade tissue barriers. Studies in acute promyelocytic leukemia cell line NB4 also show that short-term RA treatment promotes migration through activation of $\beta 2$ integrins, while, in pancreatic cancer cell line Capan-1, RA along with FGF1 induces invasion (Zang *et al*., 2000; Leelawat *et al*., 2005).

To test the hypothesis that short-term RA treatment might positively influence migration and invasion of neuroblastoma cells, two 'N'-type neuroblastoma cell lines SH-SY5Y and IMR-32 were used in this study. These two cell lines possess tumorigenic potential, but also differentiate when treated with RA for longer periods (Phuangsab *et al*., 2001; Eggert *et al*., 2002). Our results showed that short-term treatment with RA promoted migration and invasion of SH-SY5Y cells, but not IMR-32 cells. The different responses to RA in these two cell lines may be due to the different activation of RARs and RXRs.

Results

Short-term RA treatment is not sufficient to block proliferation of neuroblastoma cells

To determine the effect of RA on cell proliferation, SH-SY5Y and IMR-32 cells were treated with or without RA for 5 days, and changes of cell number and [^{3}H]thymidine incorporation were analysed. Compared to untreated cells, 1–3 days of RA treatment had no significant effects on cell number (Figure 1a and c) and [^{3}H]thymidine incorporation (Figure 1b and d). RA significantly inhibited the increased cell number and [^{3}H]thymidine incorporation from 4 days of treatment in SH-SY5Y and IMR-32 cells. RA induces expression of TGase, a dual-function G protein showing GTP binding/hydrolysing as well as transamidation functions that is involved in a number of cellular signaling events (Smethurst and Griffin, 1996; Folk and Finlayson, 1977; Fesus and Piacentini, 2002; Rodolfo *et al*., 2004). Hence, we studied expression of TGase after RA treatment. RA-induced sustained TGase expression as early as 24 h of treatment in SH-SY5Y cells, and the expression of TGase was observed from 4 days of treatment in IMR-32 cells (Figure 1e).

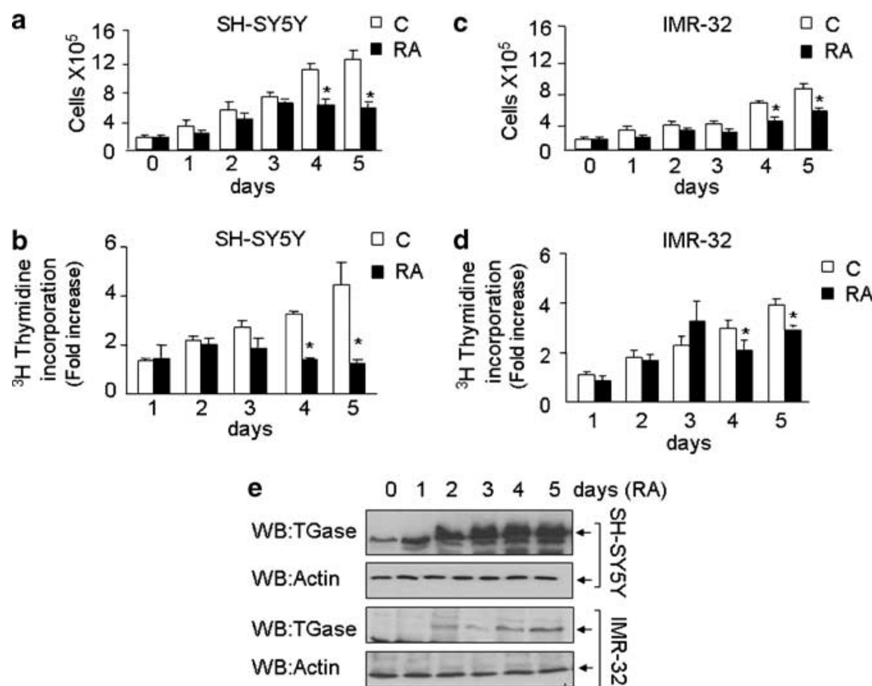

Figure 1 Effect of RA on proliferation and TGase expression in neuroblastoma cells. SH-SY5Y and IMR-32 cells were cultured in 24-well plates, treated with or without RA (5 μM) for 5 days. Cell number was counted by a hemacytometer (**a**, **c**). [^{3}H]thymidine (1 μCi/ml) was added 4 h before cell harvest. The total radioactivity of incorporated [^{3}H]thymidine was measured by liquid scintillation counting (**b**, **d**). Each *bar* represents the mean ± s.e. of three separate experiments. *$P<0.05$ versus untreated control. RA induces TGase expression. Total cellular proteins (50 μg) were separated on SDS–PAGE and blotted with anti-TGase antibody or with antiactin for normalization (**e**). The data are representative of three separate experiments.

Short-term RA treatment promotes migration and invasion of SH-SY5Y cells

The effects of RA on cell migration and invasion potential were determined. SH-SY5Y cells were treated with RA for 5 days, and the number of migrating cells was analysed using transwell polycarbonate inserts (8 μm pore size, 10 000 pores/cm^2). RA increased cell migration by 4–5-fold after 3 days of treatment, and the number of migrating cells reduced to 2–3-fold after 4–5 days of treatment (Figure 2a and b). Next, the effect of RA on capacity of the cells to invade tissue barriers was studied in an *in vitro* invasion assay, using matrigel matrix-coated transwell inserts. RA treatment for 1–2 days increased invasion capacity to about 2–3-fold (Figure 2c and d), while the invasion capacity was significantly inhibited by 5 days of RA treatment. Compared to SH-SY5Y cells, short-term RA treatment was not effective in inducing migration or invasion in IMR-32 cells (Figure 3a–f).

RA induces expression and activation of RARs and RXRs

RARs and RXRs, specially RAR/RXR heterodimers, mediate RA effects (Sporn *et al*., 1994). To study the role of RAR and RXR in RA-induced migration and invasion, the expression and activation of RARs and RXRs in RA-treated SH-SY5Y and IMR-32 cells were determined. In SH-SY5Y cells, RA significantly induced expression of RARα, β and RXRγ and RARβ and RXRβ in IMR-32 cells, while a slight increase in RARα, γ and RXRα expression was also observed in IMR-32 cells (Figure 4a and b). DNA-binding activity of RAR and RXR was determined by electrophoretic mobility shift assays (EMSA), using oligonucleotides corresponding to the RAR- or RXR-binding site, as described previously (Ishaq *et al*., 2000). The DNA–protein complex with RAR and RXR was observed after 1 day of RA treatment, and sustained to 3 days in SH-SY5Y cells (Figure 4c). In IMR-32 cells only a transient activation of RAR was observed (Figure 4d), while RXR was activated after 24 h of RA treatment (Figure 4d).

Role of RARs and RXRs in RA-induced migration and invasion in SH-SY5Y cells

We first studied the involvement of RAR/RXR in RA-induced TGase expression in SH-SY5Y cells. RA, 4-(E-2-[5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl]-1-propenyl) benzoic acid (TTNPB) (RAR agonist) and 9-*cis*-RA (RAR/RXR agonist) increased expression of TGase, while RXR agonist AGN194204 alone was not able to induce TGase expression. The increased expression of TGase by RA was inhibited by RAR antagonist AGN193109 (Figure 5a). Further, the role of RARs in migration and invasion was studied. TTNPB and 9-*cis*-RA increased migration and invasion 2–4-fold, compared to untreated control (Figure 5b and c), while AGN194204 did not affect migration and invasion capacity. RA-induced migration and invasion was blocked by AGN193109. These results indicated that RARs were involved in regulating RA effects on cell migration and invasion.

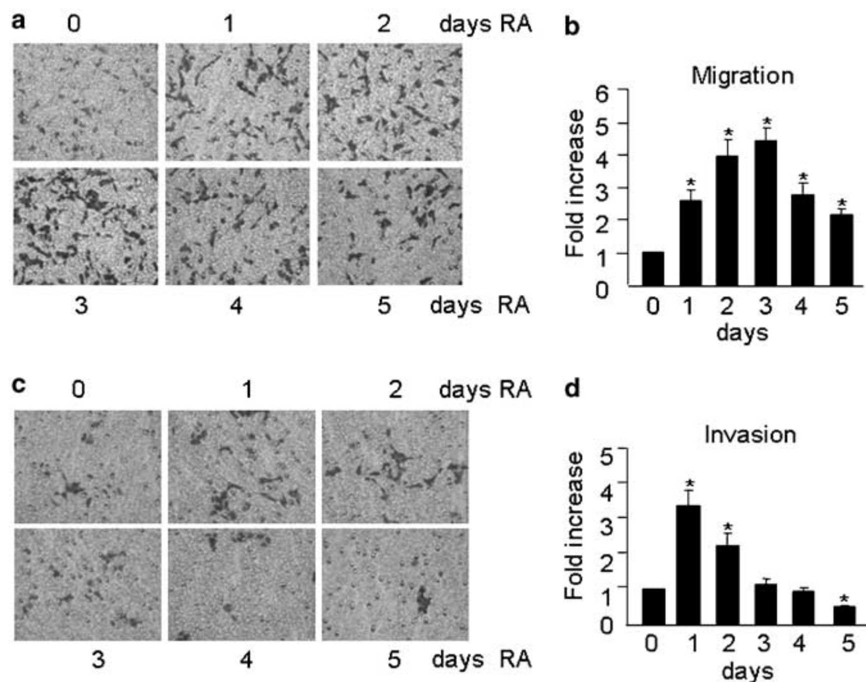

Figure 2 Migration and invasion of RA-treated SH-SY5Y cells. RA-treated SH-SY5Y cells were allowed to migrate on collagen IV-coated transwell inserts for 12 h, stained and photographed (**a**). Average number of migrated cells from **a** (**b**). Invasion of RA-treated SH-SY5Y cells on matrigel matrix-coated transwell inserts. Cells invaded through the membrane after 48 h of incubation were stained and photographed (**c**). Average number of cells from **c** (**d**). *$P<0.05$ versus control.

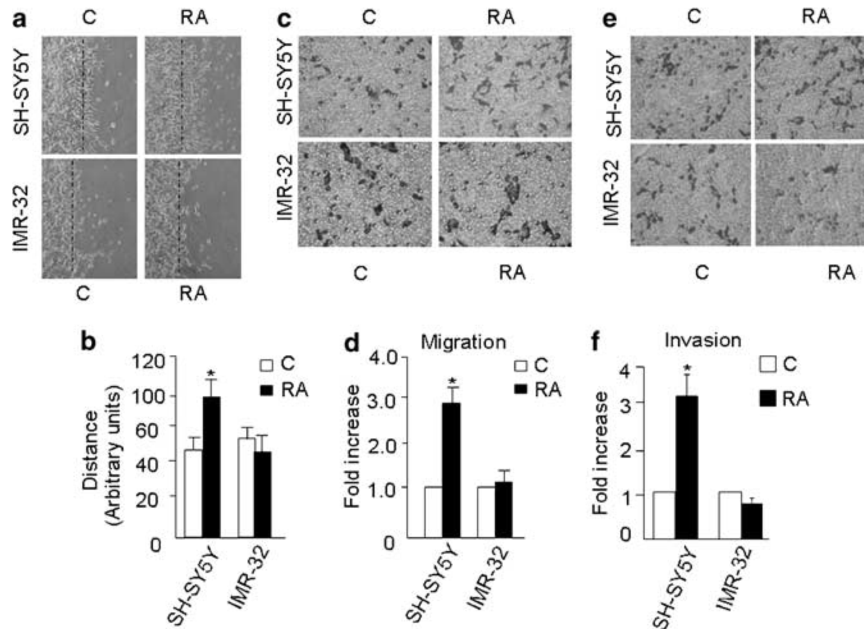

Figure 3 Different effects of RA on migration of SH-SY5Y and IMR-32 cells. Migration of untreated (**C**) and RA-treated (24 h) SH-SY5Y and IMR-32 cells in a wound-healing assay 24 h after wounding (**a**). The dotted line indicates wound edge. Distance migrated by cells from **a** (**b**). Results are mean ± s.e. of two separate experiments with three replicates each. *$P<0.05$ versus control. SH-SY5Y and IMR-32 cells were treated with or without RA for 24 h, and migration assay was performed (**c**). Average number of migrated cells from **c** (**d**). *$P<0.05$ versus control. Invasion assay was performed in untreated and RA-treated (24 h) SH-SY5Y and IMR-32 cells (**e**). Average number of invaded cells from **e** (**f**). *$P<0.05$ versus control.

RA-induced TGase is necessary for migration and invasion

To study the role of TGase in migration and invasion, adenovirus-mediated overexpression of wild-type TGase (TG-WT) was used. Significantly increased expression and activation of TGase was observed in TG-WT-infected cells (Figure 6a and b). TGase overexpression did not significantly affect migration or invasion in SH-SY5Y and IMR-32 cells (Figure 6c and d). However, overexpression of antisense TGase, which blocks expression of TGase in SH-SY5Y cells (Figure 7a), prevented RA-induced migration and invasion (Figure 7b and c), indicating that TGase is involved and mostly in cooperation with some other protein(s) mediate RA-induced migration and invasion. It has been shown that PI3K signaling is involved, in RA-mediated cell differentiation and migration (Pola *et al*., 2003; Pan *et al*., 2005). Using PI3K inhibitor LY294002, the role of PI3K in RA-induced migration and invasion was determined. Opposite to our anticipation, LY294002 had no effect on RA-induced migration or invasion in SH-SY5Y cells (Figure 7d and e), indicating that PI3K signaling is not involved in RA-induced migration and invasion of SH-SY5Y cells.

Discussion

Neuroblastoma is a heterogeneous tissue consisting of neuronal (‘N’ type), schwannian (‘S’ type) and intermediary (‘I’ type) cells. Of these different cell types, only the ‘N’-type cells possess tumorigenic potential and play an important role during tumor metastasis (Biedler *et al*., 1988; Ciccarone *et al*., 1989). Effects of RA on ‘N’-type neuroblastoma cells are variable; the RA-sensitive cells differentiate, quit cell cycle and migrate to form cell aggregates resembling ganglia, while differentiation-resistant cells show increased invasion (Chambaut-Guerin *et al*., 2000; Farina *et al*., 2002). Also, under normal conditions, RA plays an important role in the development of nervous system and migration of neural crest cells (Maden and Holder, 1992). It is therefore interesting to study RA effects on migration of neuroblastoma cells which originate due to improper differentiation of neural crest cells. Also, understanding whether duration of RA treatment plays any role is important because retinoids are used to treat neuroblastomas (Reynolds *et al*., 2003).

In SH-SY5Y cells, longer RA treatment is previously shown to inhibit invasion; however, effects of short-term RA treatment are not known (Voigt and Zintl, 2003). Our data show that short-term RA treatment is not sufficient to induce complete differentiation, but induces migration and invasion. RA-induced migration could thus represent a step towards differentiation by formation of ganglionic cell aggregates, or it could be due to alteration in some cellular functions associated with undifferentiated cells. Further dissection of RA signaling components in SH-SY5Y cells indicates that RA effects are mediated through activation of RARs. In neuroblastomas retinoid-induced differentiation occurs

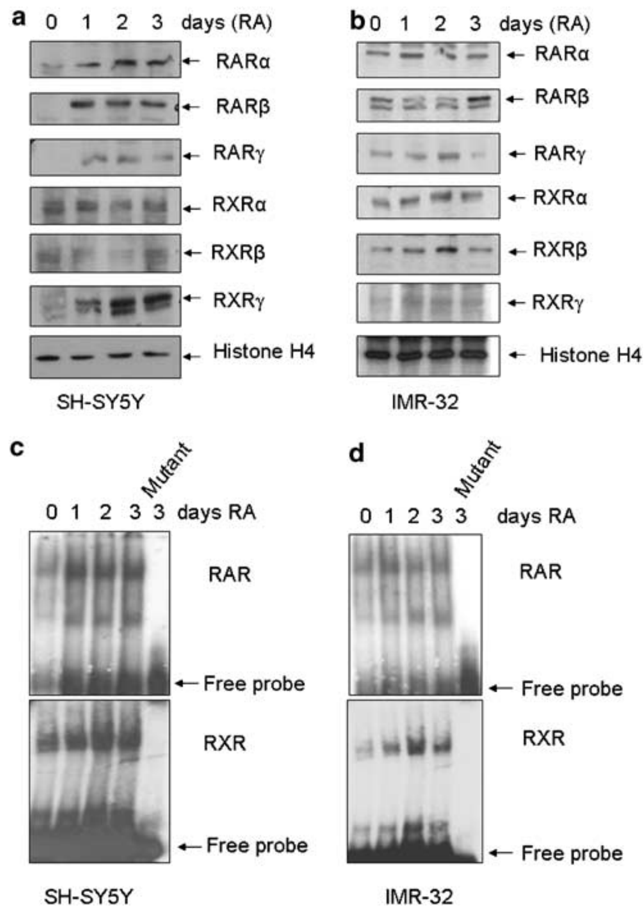

Figure 4 RA induces expression and activation of RARs and RXRs. Expression of RARs and RXRs in SH-SY5Y (**a**) and IMR-32 cells (**b**). Nuclear proteins (20 μg) were separated on SDS–PAGE and blotted with antibodies of different receptor isoforms or antihistone antibody for normalization. Activation of RARs and RXRs in SH-SY5Y (**c**) and IMR-32 cells (**d**). EMSA was performed using 10 μg nuclear proteins as described in 'Materials and methods'. As a control, mutant RAR or RXR oligonucleotides were used (lanes marked mutant).

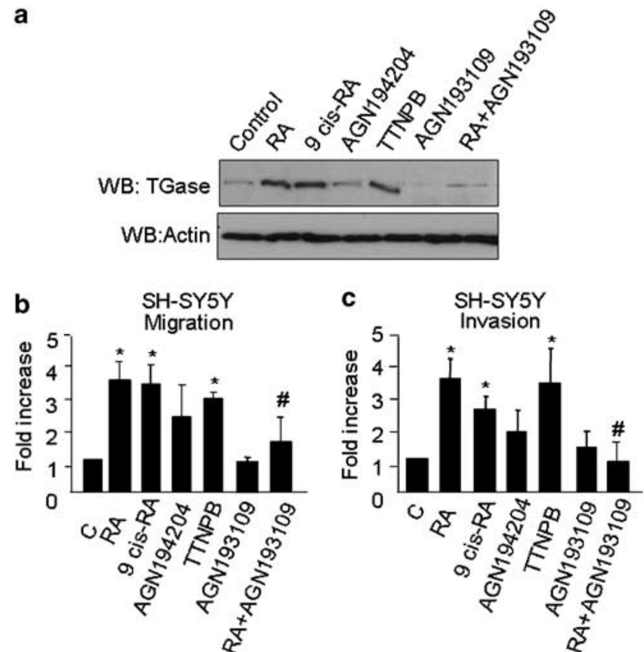

Figure 5 Effect of RAR/RXR agonists and antagonists on migration and invasion of SH-SY5Y cells. SH-SY5Y cells were treated with RA, TTNPB (1 μM), AGN194204 (1 μM) and 9-*cis*-RA (1 μM) for 24 h, or treated with RAR antagonist AGN193109 (5 μM) for 1 h and then treated with or without RA for an additional 24 h. Total cellular proteins (50 μg) were separated on SDS–PAGE and blotted for TGase (**a**). For normalization, blots were re-probed with antiactin antibody. Under similar treatment conditions with **a**, cell migration (**b**) and invasion assay (**c**) were performed as described in Figure 2. $^{*}P<0.05$ versus control, $^{\#}P<0.05$ versus RA.

through RAR/RXR heterodimer activation, while alteration in RAR function alone is associated with cancerous growth (Carpentier *et al*., 1997; Giannini *et al*., 1997; Niles, 2004). Thus, higher level of RAR activation observed in SH-SY5Y cells, compared to IMR-32 cells, might be responsible for migration and invasion. Another reason could be increased expression of TGase in SH-SY5Y cells compared to IMR-32 cells. Using agonists and antagonists of RAR and RXR, our studies show that activation of RAR results in increased TGase expression, migration and invasion. Although previous studies have shown involvement of RAR and RXR in TGase expression, our findings suggest that expression of TGase occurs through activation of RAR-dominated pathways (Floyd and Jetten, 1989; Denning and Verma, 1991; Davies *et al*., 1992; Zhang *et al*., 1995).

In SH-SY5Y cells, overexpression of TG-WT had no effect on migration or invasion, while expression of antisense TGase blocked RA-induced migration and invasion. On the other hand, inhibition of PI3K with LY294002 did not affect RA effects on migration and invasion. A promigratory role of TGase is known in breast cancer cells, $CD8^{+}$ T cells and retinal pigment epithelial cells, while, in a recent study, GTPase function of TGase inhibited motility of aortic smooth muscle cells through interaction with the cytoplasmic tail of integrin α subunit (Mohan *et al*., 2003; Kang *et al*., 2004; Mehta *et al*., 2004; Priglinger *et al*., 2004). Interaction between surface TGase and integrins and proteins in ECM such as fibronectin are known to play an important role in adhesion and migration (Juliano, 1994; Isobe *et al*., 1999; Akimov *et al*., 2000; Praise *et al*., 2000; Abel, 2001; Akimov and Belkin, 2001). In addition to TGase, RA also activates ERK1/2 MAP kinases, metalloproteinases and integrins in neuroblastomas, which might also contribute to migration and invasion (Rossino *et al*., 1991; Kim *et al*., 1997; Farina *et al*., 1999; Abel, 2001; Singh *et al*., 2003; Stahle *et al*., 2003). Indeed a positive correlation has been observed in expression of metalloproteinases and aggressiveness of neuroblastomas and other human malignancies (Mackay *et al*., 1992; Sato *et al*., 1995; Festuccia *et al*., 1996; Sugiura *et al*., 1998). Thus, it is possible that proteins such as integrins which are involved in migration cooperate with TGase to induce migration and invasion.

In conclusion, our studies demonstrate that RA responses vary in SH-SY5Y and IMR-32 neuroblasto-

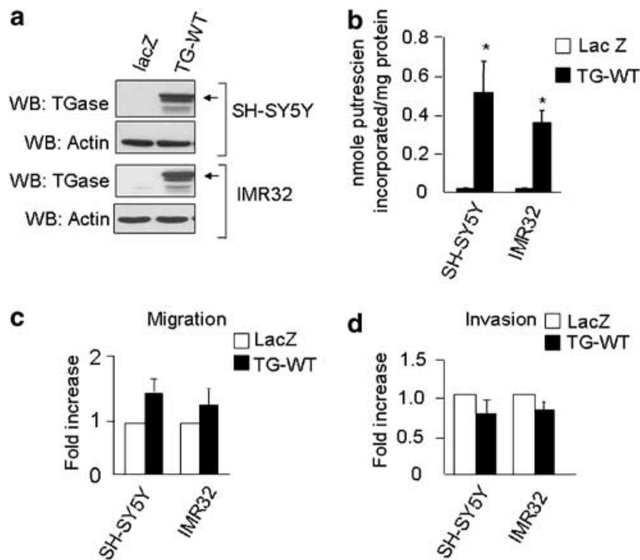

Figure 6 TGase overexpression does not affect migration or invasion. SH-SY5Y and IMR-32 cells were infected with adenovirus-mediated overexpression of wild-type TGase (TG-WT) or lacZ at 25 MOI for 24 h. TGase expression was determined as described in Figure 1e (**a**). SH-SY5Y and IMR-32 cells were infected with TG-WT or lacZ at 25 MOI for 24 h. Transamidation activity was determined using 200 μg total cellular proteins as described under 'Materials and methods' (**b**). *$P<0.05$ versus lacZ control. After infection with TG-WT or LacZ at 25 MOI for 24 h, the effect of overexpression of TGase on cell migration (**c**) and invasion (**d**) was determined.

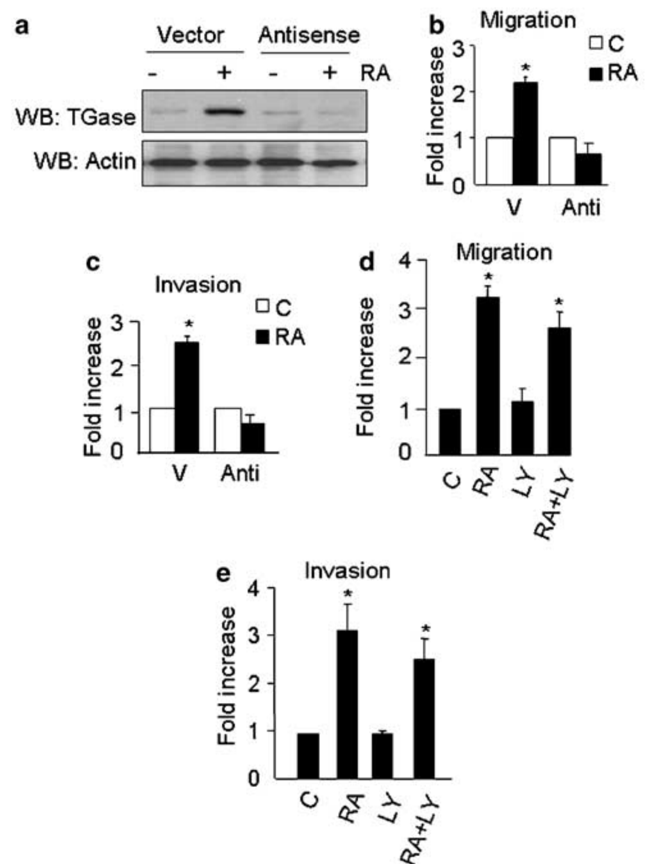

Figure 7 RA-induced TGase expression but not PI3K activation is necessary for RA-induced migration and invasion. SH-SY5Y cells stably expressing antisense TGase (Anti) or empty vector were treated with RA for 24 h. Expression of TGase was examined as described in Figure 1e (**a**). The role of TGase in RA-induced cell migration (**b**) and invasion (**c**) was determined. *$P<0.05$ versus RA untreated control. SH-SY5Y cells were treated with RA for 24 h in the presence and absence of LY294002 (10 μM). Role of PI3K in RA-induced cell migration (**d**) and invasion (**e**) was determined. *$P<0.05$ versus RA untreated control.

ma cell lines. These differences could be due to the different levels of activation of RAR and RXR receptors. Also, the duration of RA treatment might determine the outcome; short-term RA treatment promotes migration and invasion, while long-term RA treatment leads to differentiation. These studies also demonstrate that TGase is required for mediating short-term RA effects on cell migration; however, additional molecules such as integrins, metalloproteinases and ERK1/2 MAP kinases might also be involved.

Materials and methods

All *trans*-RA, 9-*cis*-RA, TTNPB and other common chemicals were purchased from Sigma (St Louis, MO, USA). AGN194204 and AGN193109 were from Allergan (Irvine, CA, USA). Tissue culture media and serum were purchased from Invitrogen (Carlsbad, CA, USA). Matrigel matrix and collagen IV were from BD biosciences (Franklin Lakes, NJ, USA). TGase antibody was from Neomarkers (Newington, NH, USA), and antibodies against actin, RAR and RXR isoforms as well as RAR, RXR oligonucleotides were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Stable SH-SY5Y cells expressing antisense-TGase were a kind gift from Dr GV Johnson (University of Alabama at Birmingham, AL, USA).

Cell culture and RA treatment

SH-SY5Y and IMR-32 cells were grown in medium containing 10% serum, 1% streptopenicillin at 37°C in a humidified incubator with 5% CO_2. The cells were incubated in medium containing 3% serum for 12 h and then treated with 5 μM RA or vehicle (DMSO) for 5 days. Treatment of RAR or RXR agonists and antagonists was performed in medium containing 3% serum.

Construction of TGase adenovirus

TGase virus was constructed using BD Adeno X expression system I (BD Biosciences) according to the manufacturer's protocol. Briefly, TG-WT cDNA cloned in pcDNA3.1 vector was first subcloned in pshuttle 2 vector and then in BD adenoX adenoviral vector. HEK293 cells were transfected with recombinant adenoviral plasmid for packaging of adenovirus particles. Adenovirus was purified on CsCl gradient and used for experiments at 25 MOI. Cells infected with lacZ adenovirus were used as control.

Transglutaminase assay

Transglutaminase assay was performed as described previously using cell lysates from adenovirus-infected cells (Singh *et al.*, 2003).

Cell proliferation assay
Cells (2×10^5/well in 24-well plates) were treated with or without RA, and cell proliferation profile was monitored by counting cells each day using a hemacytometer. [^{3}H]thymidine incorporation was studied as described before (Arvidsson *et al*., 2001). The experiments were repeated three times with four replicates each.

Cell lysis and Western blot analysis
Total cellular proteins were isolated and Western blotted as described previously (Singh *et al*., 2003). Nuclear proteins were isolated using a nuclear isolation kit (Sigma, St Louis, MO, USA) and used for Western blotting. Protein concentration was determined using a protein estimation kit (Bio-Rad, Hercules, CA, USA).

Electrophoretic mobility shift assay (EMSA)
EMSA was performed using 10 μg nuclear proteins as reported previously (Carpentier *et al*., 1997; Palm-Leis *et al*., 2004). Binding-deficient RAR and RXR mutant oligonucleotides were used as negative control.

Wound-healing assay
RA-induced motility was studied in a wound-healing assay as described previously (Voigt and Zintl, 2003).

Migration assay using transwell plates
Cell migration was also studied using transwell inserts coated with 10 μg/ml collagen IV as a chemoattractant. Trypsinized cells were suspended in serum-free medium without RA and 5×10^5 cells were added to the upper chamber of transwell inserts. Medium with 3% serum was added to the lower chamber. After 12 h incubation for SH-SY5Y cells or 5 h for IMR-32 cells, nonmigrated cells on the upper surface of the membrane were scrapped off, cells on the lower surface were stained using the Hema 3 staining system (Fisher Scientific, Houston, TX, USA), photographed ($\times 40$ magnification) and counted from 10 randomly selected fields. All the experiments were repeated at least three times with two replicates each.

Cell invasion assay
Cell invasion was studied using matrigel-coated (0.7 mg/ml) transwell inserts as described previously (Mehta *et al*., 2004). Invasion assay was performed similar to migration assay, only the cells were incubated for 48 h. All the experiments were repeated at least three times with two replicates each.

Statistical analysis
Statistical significance of the data was evaluated using one-way analysis of variance, with a probability level 0.05 indicating significant differences.

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