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Expression of Co-factors (SMRT and Trip-1) for Retinoic Acid Receptors in Human Neuroectodermal Cell Lines

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Retinoic acid (RA) induces growth inhibition, differentiation or cell death in many human neuroblastoma cell lines. Recently, the transactivation activity of nuclear retinoids receptors has been shown to be modulated through physical association with other proteins that act as co-activators or as co-repressors. We investigated the expression of the co-repressor (SMRT) and co-activator (Trip 1) for retinoid and thyroid-hormone receptors in several neuroectodermal tumour cell lines, and its modulation by all-*trans*-retinoic acid, as well as by synthetic agonists, for RARα, RARβ, RARγ and RXR. We demonstrate that (i) SMRT and Trip-1 mRNAs are expressed in many human neuroblastoma and melanoma cell lines in basal conditions, (ii) SMRT mRNA expression in human neuroblastoma cell line SK-N-BE(2) increases after 48 hours of incubation with 1 μM RA and RARs specific agonists, (iii) Trip-1 mRNA in the same cell line does not change during incubation with RA or selective synthetic agonists for RARs and RXR.

The effects of retinoids are modulated by the interaction of many factors at different steps of their pathway. A first level of complexity in retinoids signals is due to "multiplicity" of retinoids obtained intracellularly by oxidative and isomerization reactions (all-*trans*-RA, didehydro RA, 9-*cis*-RA). The cellular retinoic acid binding proteins (CRABP I and II), are cytosolic proteins whose gene expression is induced by RA (1,3) and which bind all-*trans*-RA with high affinity, finely modulating its intracellular concentration. Two families of nuclear retinoic acid receptors, each consisting of three receptors types α, β, γ, have been discovered: retinoid acid receptors (RARs) and retinoid X receptors (RXRs) (4-6). All RARs receptors are present in different isoforms (7,8) and bind to RA responsive elements (RARE) as heterodimers with RXRs (4). This complex network of factors is further complicated by the recent discovery by the two-hybrid screening system of a retinoic acid and thyroid-hormone receptor associated co-repressor (TRAC): the silencing mediator for retinoic acid and thyroid hormone receptors (SMRT) (9) also called T_3 receptor associating cofactor-2 (TRAC-2) (10). This co-repressor preferentially complexes to RAR and marginally with RXR. Binding of ligand to RAR reduces the interaction with SMRT but not with RXR, and the binding of ligand to RXR has no effect. The preferential interaction of SMRT with RAR in the absence of a ligand suggest its role in mediating a trascriptional silencing effect: the release of SMRT may be a prerequisite for ligand-dependent transactivation. A role of the polarity of the responsive element in mediating the silencing effect has been postulated for a similar nuclear receptor co-repressor (N-CoR), which shows 41% aminoacid identity with SMRT (11). RAR/RXR heterodimers activate transcription in DR+5 elements (with RAR in 3′ position) in response to RAR-specific ligand (all-*trans*-RA), while they do not activate DR+1 repeats (with RAR in 5′ position); in the latter configuration, the co-repressor is unable to dissociate from the complex, thereby preventing transcriptional activation (12,13).

Factors, referred to as co-activators, are thought to serve as bridging molecules between the receptors and the basal transcriptional machinery, thus mediating activation of transcription. A candidate human co-activator that interacts with both the thyroid-hormone receptor and the retinoid-X-receptor in a ligand-dependent manner has recently been identified : thyroid hormone receptor interacting protein (Trip 1) (14). The

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complex biology of the action of retinoids depends, thus, on the different ligands, DRs repeats, co-factors, and RA receptors present.

Retinoids are known to modulate cell death, differentiation and proliferation in a variety of neoplastic cell types, including neuroblastoma. In the human neuroblastoma cell line SK-N-BE(2), RA induces differentiation and growth inhibition (15). We have previously shown the presence of the RA receptor pattern in this cell line. In particular, RARα and γ, RXR α and γ were all constitutively expressed, while RARβ was only expressed after RA incubation (16).

The aim of our study was to investigate the presence of SMRT and Trip-1 expression in neuroblastoma and melanoma cell lines and their possible modulation by retinoids in the human neuroblastoma cell line SK-N-BE(2).

MATERIALS AND METHODS

Reagents. Plastics, tissue culture media, trypsin, EDTA, HEPES, L-glutamine, sodium bicarbonate, phosphate-buffered saline, fetal bovine serum, and non-essential amino-acids were obtained from Flow Laboratoires Ltd. (Herts, United Kingdom). The synthetic RA analogues were supplied by CIRD-GALDERMA Company (Sophia-Antipolis, France). We used CD 14 (*all*-trans-RA); CD336 (Am 580), that selectively binds to RARα; CD666, an agonist for RARγ; CD2314, an agonist for RARβ; CD2624, an RXR-selective agonist (16). All five compounds were stored in DMSO at -20°C.

Cell culture. The human neuroblastoma cell line SK-N-BE(2) has been described by Ciccarone et al (17). Cells were grown in monolayer culture in a 1:1 mixture of MEM and Ham's F-12 media supplemented with 15% heat-inactivated fetal bovine serum, sodium bicarbonate (1.2mg/ml$_{-1}$), HEPES buffer (15 mM), L-glutamine (2 mM), and non-essential aminoacids (1%v/v). Cells were fed every 3-4 days and split weekly at a ratio of 1:5-1:10 using trypsin (0.025%)-EDTA (0.02%). Cells were routinely fed 24 h before being harvested for experiments. The cells were incubated 48 h, with 1μM *all*-trans-RA or synthetic retinoid analogues (5mM stock solution in 70% ethanol); 0.07% ethanol was added to the control cultures. After removal of the culture medium, cells were centrifuged at 800 $\times$ g for 10 minutes and then immediately subjected to RNA extraction.

RT-PCR analysis. Total RNA was purified using a standard method and reversed transcribed with AMV reverse transcriptase, according to the protocol provided by Promega Co. (Madison, WI, USA). The newly transcribed cDNA was amplified by differential-PCR using β2-microglobuline as internal standard whose expression is not modulated by RA. Kinetics and titration analysis were performed to determine the conditions in which data could be obtained before the amplification reactions reached the plateau phase. PCR was performed with a DNA Thermal cycler (Perkin-Elmer Catus). PCR for SMRT was carried out after a pre-heating step at 94°C for 5 minutes through 32 cycles (denaturation at 94°C for 1 minute, annealing at 61°C for 1 minute and extention at 72 °C for 1 minute) and a final extention at 72°C for 7 minutes. The reaction mixture for a final volume of 50 μl was as follows: 1 μl of the RT reaction mixture, PCR buffer 10 mM Tris (pH 8.3), 50 mM KCL, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.25 units AmpliTaq DNA polymerase, and 0.5μM of the listed primers:

-SMRTas: 5′-AGGCGTCGACGTCAGCTTTCGGT-3′

-SMRTs: 5′-TCATTGTGCCTGAGCTGGGTAAG-3′

-Trip-1s: 5′-ATGACAGCTACACTCTGCACA-3′

-Trip-1as: 5′-CAATTCAGAGCCAGAGACAC-3′.

-β2-microglobuline-as: 5′-ATCTTCAAACCTCCATGATG-3′

-β2-microglobuline-s: 5′-ACCCCCACTGAAAAAGATGA-3′.

β2-M primer sequences generated a 110 bp product. Primer sequences for SMRT were drawn to obtain the amplification of a 184 bp fragment (bp 1517-1701) from its published sequence (9) (Gene Bank accession number, U37146).

The PCR for Trip-1 was performed in the same conditions except for the annealing temperature (58 °C), the number of cycles (30) and the dilution of the template (1:3), as suggested by the dilution and kinetic curves. The Trip-1 primers were drawn to obtain the amplification of a 305 bp fragment (bp370-675) from its published sequence (Gene Bank accession number, L38810).

RT-PCR product (20 μl) were separated on 2% agarose, 0.01 % ethidium bromide gels and visualized on a transilluminator. The ratio of the target to control β2-M gene products was determined by bidimensional densitometry on a GS-670 Densitometer (BioRad). The PCR products obtained during kinetics and titration analysis were also quantitated by HPLC.

HPLC analysis. The RT-PCR product analysis was performed by HPLC according to van Hille (18). Briefly 20 μl from each PCR reaction were injected into a anion exchange TSK-Gel DEAE-NPR column (Montgomeryville, PA). The mobile phase consisted of various volume proportions of buffer A (0.025 M Tris-HCl pH 9) and buffer B (0.025 M Tris HCl pH 9, NaCl 1M). The ratio A/B changed in the gradient program as follows: 0.01 min 70/30; 0.1 min 60/40; 3 min 48/52, 6.5 min 28.4/71.6, 6.51 min 0/100. , 8-10 min 70/30. Flow rate 1 ml/min. The retention time of SMRT was 6.51 minutes. The amount of PCR product was determined as Area Under Curve (AUC). All experiments were performed in duplicate.

RESULTS

Expression of SMRT and Trip-1

The analysis of the PCR product shows a high correlation between bidimensional densitometry and HPLC ($r=0.83$, $p=0.0001$).

To confirm that the PCR product of 184 bp was homologous with the SMRT fragment (bp 1517-1701), we used restriction enzyme digestion. The amplified cDNA was digested with three restriction enzymes (Fok I, Hha I, Hpa II), each of which has one restriction site within the amplified sequence. All enzymes produced fragments of the predicted size (fig.1A).

Restriction enzyme digestion was also performed to confirm that the PCR product of 305 bp was homologous with the Trip fragment (370-675). The amplified cDNA was digested with three restriction enzymes (RSA I, Mnl I, Fok I). All enzyme produced fragments of the predicted size (fig.1B).

We detected a basal expression of SMRT and Trip-1 mRNAs in SK-N-BE(2), KCNR, IMR-32 human neuroblastoma cell lines as well as in SK-mel-28, 12443 and other three yet unpublished human melanoma cell lines established in our Institute (Dr. S.D'Atri IDI-IRCCS, Rome) (Gar, Con, Par) (fig. 2A and B).

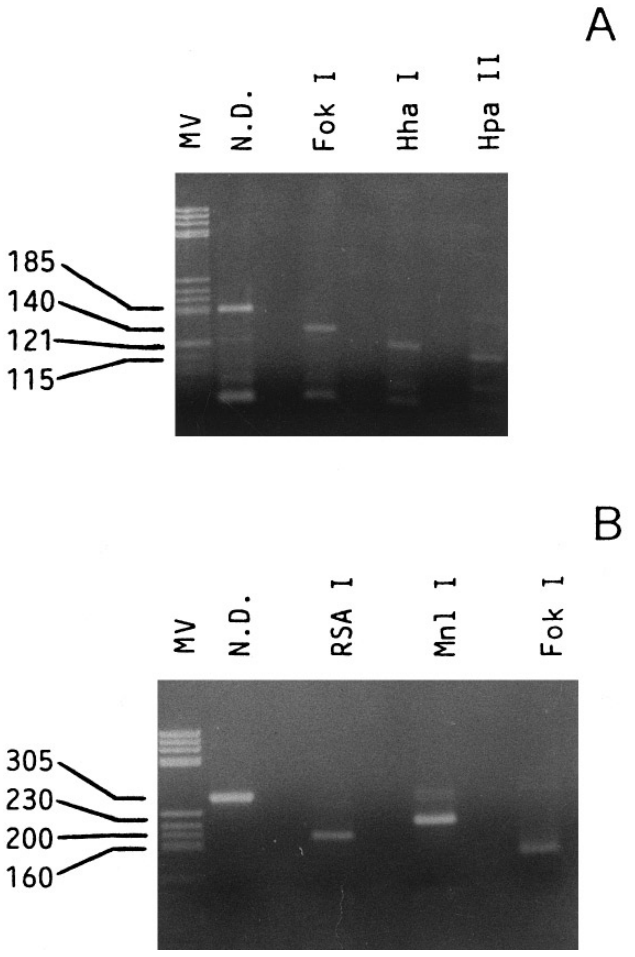

FIG. 1. Panel A, RT-PCR for SMRT: restriction enzyme digestion (N.D. = non digested sample). Panel B, RT-PCR for Trip-1:restriction enzyme digestion (N.D. = non digested sample).

Regulation by Retinoids in SK-N-BE(2) Neuroblastoma Cell Line

In order to determine whether RA modulates SMRT and Trip-1, we used retinoid receptor selective agonists, used in our previous paper (16). We performed these experiments in the SK-N-BE(2) neuroblastoma cell line whose RA-receptor pattern as well as RA-responsiveness are yet to be studied in our laboratory (15, 16).

Using the β2-M gene as an internal standard in a differential RT-PCR, we compared the expression of SMRT and Trip-1 after 48 hours of incubation with *all*-trans-RA (1 μM) with a corresponding 48-hour untreated control. In the same experiment, we studied the modulation of SMRT (fig.3A) and Trip-1 (fig. 3B) expression in our cell line using synthetic agonists for RARα, RARβ, RARγ and RXR at the same concentration. The ratio SMRT/β2-M and Trip-1/β2-M was used to compare the different lines (fig.3C). RA and RARs agonists slightly upregulate SMRT expression, whereas the RXR agonist shows no effect. Trip-1 expression was not modulated by RA or synthetic agonists.

DISCUSSION

Although, the effects of retinoids involve their interaction with nuclear RARs/RXRs heterodimers, they are subject to a complex control through interactions with co-factors acting both as transcription activators and repressors. In the absence of ligands, some "transcription silencing factors" may associate with unliganded receptor heterodimer, interfering with the basal transcription machinery. The addition of a ligand may result in a conformational change of particular receptor domains displacing the co-repressor and recruiting the co-activator for transactivation (11). Moreover, the co-factors may represent important targets through which non-hormonal signal transduction pathways regulate the function of nuclear receptors, independently of the ligands. The effective concentration of retinoids used to obtain inhibition of tumor cell growth *in vitro* is often not physiological. Clinicians hope that retinoids may have a potential role in chemoprevention and chemotherapy *in vivo*. Recently, 9-*cis*-RA has been shown to be more effective that all-*trans*-RA, although at high concentrations, both in inducing neuroblastoma cell differentiation *in vitro* (19,20) and in the suppression of breast cancer (21).

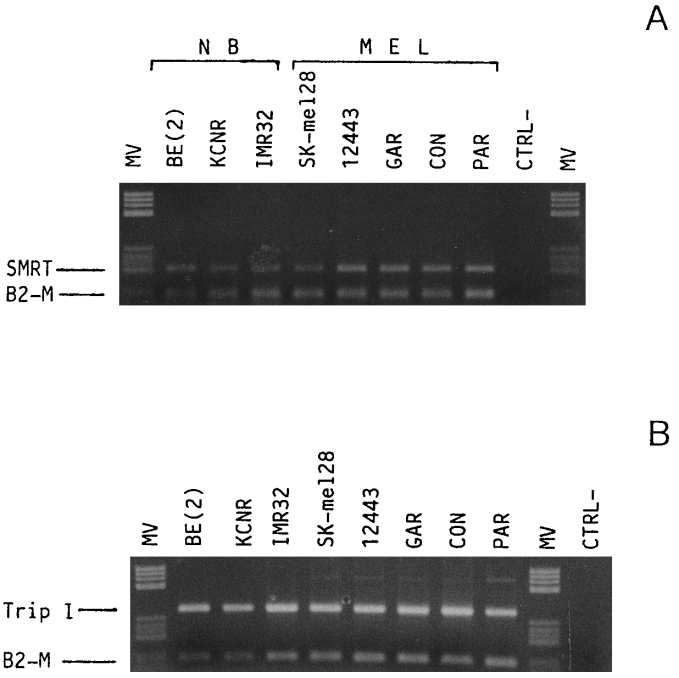

FIG. 2. Panel A, RT-PCR for SMRT: basal expression in human neuroblastoma and melanoma cell lines. Panel B, RT-PCR for Trip-1: basal expression in human neuroblastoma and melanoma cell lines.

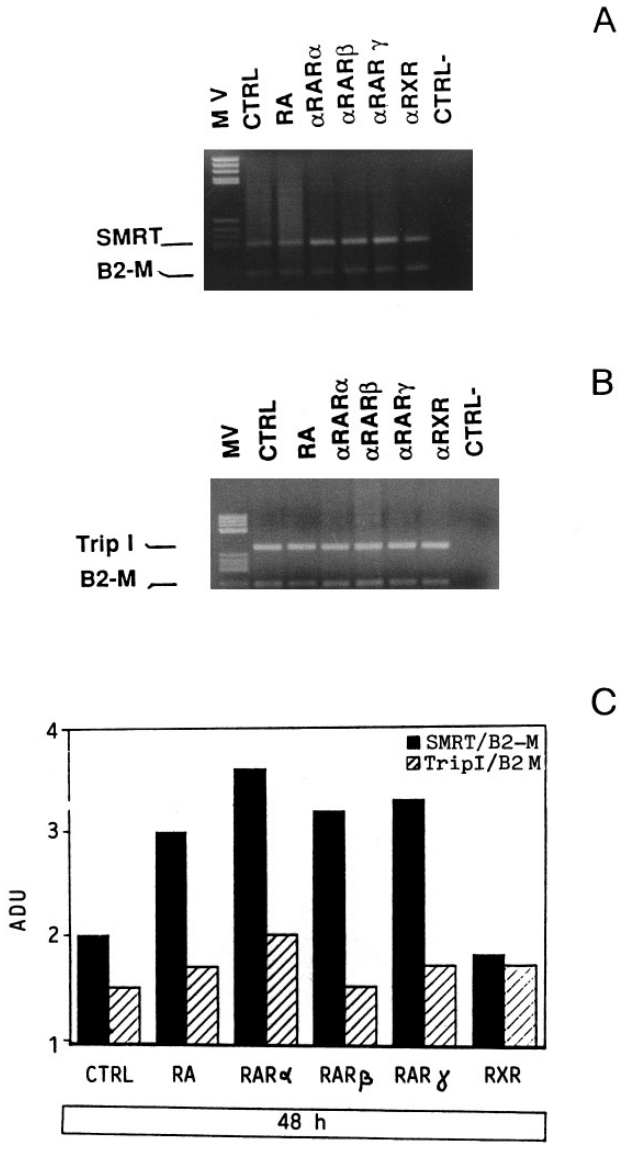

FIG. 3. RT-PCR for SMRT (panel A) and Trip I (panel B): modulation by CD14 (all-*trans*-RA), CD336 (agonist for RARa), CD666 (agonist for RARg), CD2314 (agonist for RARb), and CD2624 (agonist for RXR). Incubation with 1μM of each for 48 hours. Densitometric analysis of the RT-PCR product after incubation with RA and synthetic agonists (ratio between SMRT/β2-M and Trip I/β2-M) (panel C).

RA plays an important role in the regulation of proliferation and differentiation in neuroectodermal tumours. This effect is sometimes antagonized by growth factors, such as IGF-2 (22). Human neuroblastoma cell lines include three morphologically and biochemically distinct phenotypes (neuronal "N", intermediate "I" and flat substrate adherent "S") which undergo spontaneous transdifferentiation. Human neuroblastoma SK-N-BE(2) cells, when treated with RA, reduce their growth and differentiate toward a neuronal phenotype (N-type cells), while a subset of cells (S-type), expressing high levels of tissue transglutaminase (tTG), undergo apoptosis. RA upregulated the expression of tTG in SK-N-BE(2) cells resulting in an enhanced enzyme activity (23). The result of this is the enhanced formation of apoptotic bodies, evaluated by ultrastructure, DNA ladder, FACS analysis, Hoechst staining, $\epsilon(\gamma$-glutamyl)lysine cross-linked apoptotic bodies. Although several molecular mechanisms may be responsible of the apoptotic effect of retinoids, the upregulation of the tTG is sufficient *per se* to kill the cells, as shown in the same cell line by sense and anti-sense tTG transfection (24). The promoter of tTG contains a RARE (25) which can be activated by RARα. Since not all cell lines, and not all cells in the same cell line can be induced by retinoids into programmed cell death, we decided to evaluate the presence of co-activators or co-repressors of retinoids, that could modulate the transactivation properties.

We have shown the expression of both Trip-1 and SMRT mRNA in the human neuroblastoma cell line SK-N-BE(2). The modulation of SMRT and not of Trip-1 mRNA expression by RA and synthetic agonists seems to suggest that, in our model, the "silencing effect" plays a major role in modulating transactivation by retinoids. Considering that SMRT preferentially interacts with RARs, we can hypothesize that 9-*cis*-RA is more effective than all-*trans*-RA because its interaction with the receptor is "SMRT free".

All RARs agonists up-regulate SMRT mRNA expression, suggesting a possible positive feed-back between the ligand and the co-repressor, mediated by an RAR monomer, which should eventually stop the transactivation induced by the ligand and maintain the silence in its absence. The lack of significant modulation of SMRT mRNA expression by the RXR agonist seems to support the hypothesis that the signalling through RXR is SMRT-independent and consequently, the 9-*cis*-RA action should not be affected by this mechanism.

In conclusion, our data suggest a role for SMRT in mediating the cellular response to different retinoids and possibly in determining retinoic acid resistance in human neuroblastoma cell lines with considerable implications for retinoids potential use *in vivo.*

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