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Valproic Acid Induces the Expression of the Na^+/I^- Symporter and Iodine Uptake in Poorly Differentiated Thyroid Cancer Cells

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ABSTRACT In poorly differentiated thyroid cancer, molecular characteristics are reported to be lost such as to cause insensitivity of the tumor to radiometabolic therapy. Considerable work is in progress to identify compounds that re-differentiate thyroid cancer cells. The present study evaluates the action of valproic acid, a potent anticonvulsant recently reported to inhibit histone deacetylase, on cultured thyroid cancer cells. N-PA (poorly differentiated) and ARO (anaplastic) cells were treated with increasing valproic acid concentrations; expression of mRNA and cell localization pattern for the Na^+/I^- symporter (NIS), as well as ^{125}I uptake, were evaluated before and after treatment. Valproic acid induced NIS gene expression, NIS membrane localization and iodide accumulation in N-PA cells; it was effective at clinically-safe doses in the therapeutic range. In ARO cells, only induction of NIS mRNA was observed, and was not followed by any change in iodide uptake. Valproic acid is thus effective at restoring the ability of N-PA cells to accumulate iodide and its use in clinical trials may be recommended.

Thyroid cancer, the most frequent neoplasm of the endocrine system, which accounts for about 1% of all human cancers, is usually sensitive to conventional therapy and has a good prognosis. Unfortunately, this is not true of 30% of cases, which develop toward dedifferentiation (1). Thyroid tumors that lose differentiation characteristics, as well as those that are poorly differentiated from the start, have accelerated growth and, especially, fail to respond to traditional therapy.

The dedifferentiation process entails progressive loss of specific thyroid molecules/functions: over time, poorly differentiated thyroid cancer cells undergo a reduction/disappearance of TSH receptor (TSH-R) and thyroglobulin (TG), expression (2,3) and lose their ability to capture iodine. This last feature is closely linked to the tumor's sensitivity to radio-iodide therapy. The ability of normal thyroid cells to capture and concentrate iodine depends on the correct functioning of the Na^+/I^- symporter (NIS), (4, 5). Loss of the NIS function and/or of gene expression is a cornerstone in the dedifferentiation process (6).

Valproic acid is a potent anticonvulsant and mood stabilizer, which has recently been reported also to act as an inhibitor of histone deacetylases (7). Histone deacetylases (HDACs) regulate the histone acetylation status, which plays a crucial role in cell cycle and cancer growth (8). VPA has been shown to inhibit corepressor-associated HDACs at therapeutic concentrations, and to act as a potent inducer of differentiation in several types of transformed cells (9). Valproic acid has already been proposed for redifferentiating therapy of hematological neoplasm (9) and neuroblastoma (10), but never in thyroid cancer.

The present study evaluates the ability of valproic acid to promote re-differentiation of poorly-differentiated thyroid cancer cells, with the goal of restoring these cells' ability to capture and concentrate iodine.

Materials and methods

Cell lines and culture conditions

The papillary-thyroid-carcinoma-derived cell sub-line (N-PA) and the anaplastic thyroid carcinoma cell line (ARO) were kind gifts from Mauro Papotti and Paola Cassoni (Pathology Service, Department of Oncology, University of Turin). Cells were routinely maintained in RPMI 1640 (Sigma, St Louis, MO, USA) supplemented with 10% heat-inactivated FCS (Euroclone, Wetherby, West York, UK).

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RT-PCR of TG, TSH-R and NIS.

N-PA and ARO cells were seeded at 2×10^5 cell/well in 6-well plates; after 48 h they were treated with VPA for 48 h. Total RNA was extracted from both cell lines and from a normal human thyroid tissue sample (kindly provided by Marco Volante, Pathology Service, Department of Oncology, University of Turin) using TRIzol Reagent (Invitrogen, Groningen, The Netherlands) and following the method developed by Chomczynski and Sacchi (11). Total RNA was reverse-transcribed at 42° C for 40 min using AMV reverse transcriptase (Finnzymes, Finland) and oligodT primer (Invitrogen, Groningen, The Netherlands). The PCR reaction system contained 5μl of 10X PCR buffer, 10μl of RT product, 0.2 mM dNTP (Finnzymes, Finland), 1.25U *Taq* DNA polymerase (Finnzymes, Finland), 50 ng each of sense and antisense primers in a total volume of 50 μl. Primer for TG: 5'-GTT GGC AAC CTC ATC GTG GTC TG, 3'-AGA GAA TTC TGC AGT GCC TGG TA; primers for TSH-R: 5'-ACT TCA GAG TCA CCT GCA AGG, 3'-TTG CTA TCA GTT CCT TCA GGT G; primers for NIS: 5'-CTG CCC CAG ACC AGT ACA TGC C, 3'-TGA CGG TGA AGG AGC CCT GAA G; primers for b-actin: 5'-CTC ACC CTG AAG TAC CCC ATC G, 3'-CTT GCT GAT CCA CAT CTG CTG G. The expected PCR products were 668 bp for TG, 657 bp for TSH-R and 303 for NIS. Amplification was carried out as follows; for TG, TSH-R and β-actin: 1X3 min at 94°C; 35X30s at 94°C, 30s at 58°C, 30s at 72°C; and 1X7 min at 72°C. For NIS: 1X3 min at 95°C; 40X30s at 95°C, 1 min at 60°C, 1 min at 72°C; and 1X7 min at 72°C. PCR products were electrophoresed on 1.5% agarose gel in the presence of ethidium bromide. Gels were photographed and analyzed with the PC program Kodak 1D Image System. The net intensity of bands in each

experiment was normalized for the intensity of the corresponding β-actin band before comparison between cell lines and normal thyroid tissues.

^{125}I uptake by cells

Cells (2 x 10^4) were seeded into 24-well plates and incubated with VPA (0.5, 1, 1.5, 3 mM) for 48 or for 72 h. ^{125}I uptake was assayed as reported elsewhere, with minor modifications (12). Briefly, after aspirating drug-containing medium, the cells were washed with 1 ml HBSS (Invitrogen, Groningen, The Netherlands). ^{125}I uptake was initiated by adding 0.5 ml HBSS containing 2 μCi carrier-free Na^{125}I (Amersham Biosciences, Little Chalfont, UK) and 30 μM NaI. Incubation was for 30 min at 37°C and was terminated by removing the radioactive medium and washing the cells twice with ice-cold HBSS. Cells were then solubilized with 1 ml of absolute ethanol for 20 min, after which cell-associated iodide was measured in a γ-counter.

Immunocytochemistry for NIS

N-PA and ARO cells, grown on glass slides (Nalgene Nunc Int., Naperville IL, USA), were treated with 3 mM VPA for 72 hours; they were then fixed in 4 % paraformaldehyde. Endogenous peroxidase activity was blocked with H_2O_2 and non-specific binding sites were blocked with 10% horse serum. Cells were incubated for 1 hour at room temperature with a primary monoclonal antibody against hNIS (hNIS Ab-1, Neomarkers Fremont CA, USA) 1:400. Staining was performed with the DAKO EnVision+ System (DAB) (DAKO Corp. Carpinteria CA, USA) following manufacturer's instructions. VPA-treated cells were used as such or after permeabilization with 0.1% saponin. Horse serum was used instead of primary antibody for the negative controls.

Statistical analysis

Data are expressed throughout the text as means ± SEM, calculated from at least three different experiments. Statistical comparisons between groups were performed with analysis of variance (one-way ANOVA), the threshold of significance being calculated by the Bonferroni test. Statistical significance was attained for values of $P<0.05$.

Results

Molecular Characterization of Cell Differentiation

A sub-line of N-PA cells was chosen to study the effect of valproic acid on re-differentiation of thyroid cancer cells, since these cells do not express any of the differentiation markers we evaluated, as showed in figure 1, while the three genes are fully expressed in normal thyreocytes. As expected, their expression was completely lost in ARO cells.

Effect of VPA on NIS mRNA expression

Figure 2 shows the expression of NIS mRNA in N-PA cells. VPA was found to induce NIS expression in a dose-dependent manner; the lowest effective dose being 1 mM. VPA also restored NIS expression in ARO cells (Fig. 3), but these cells were less sensitive to the drug than N-PA cells. VPA did not induce TG or TSH-R gene expression in either N-PA or ARO cells (data not shown).

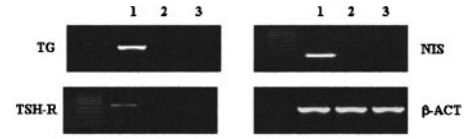

Fig. 1. Thyroglobulin (TG), TSH-receptor and NIS mRNA expression. RT-PCR for TG, TSH-R and NIS in normal human thyroid cells (lane 1), N-PA cells (lane 2) and ARO cells (lane 3). The figure shows a typical experiment.

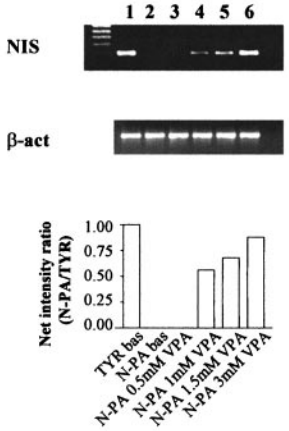

Fig. 2. NIS mRNA expression in N-PA cells after valproic acid treatment. RT-PCR for NIS and β-act in N-PA cells grown in the absence (lane 2) or presence of 0.5 (lane 3), 1 (lane 4), 1.5 (lane 5) and 3 (lane 6) mM VPA. Lower panel: semiquantitative analysis of RT-PCR results. Net intensity was determined as the ratio between N-PA cells and normal human thyroid cells (lane 1) The figure shows a typical experiment

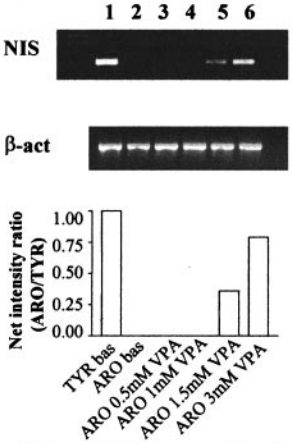

Fig. 3. NIS mRNA expression in ARO cells after valproic acid treatment. RT-PCR for NIS and β-act in ARO cells grown in the absence (lane 2) or presence of 0.5 (lane 3), 1 (lane 4), 1.5 (lane 5) and 3 (lane 6) mM VPA. Lower panel: semiquantitative analysis of RT-PCR results. Net intensity was determined as the ratio between ARO cells and normal human thyroid cells (lane 1) The figure shows a typical experiment

Effect of VPA on ^{125}I uptake

To assess whether the NIS expression induced in both N-PA and ARO cells yielded a functional NIS protein, iodine uptake by cells under VPA treatment was evaluated (Figure 4).

In N-PA cells (*A and C*) VPA-induction of NIS resulted in a progressive increase in iodine uptake, which reached maximum levels after 72 hours treatment with the highest dose of VPA used; it is interesting that at that time the increase in iodine uptake perfectly paralleled the induced NIS mRNA expression.

On the other hand, the VPA-induction of NIS in ARO cells (*B and D*) did not cause any significant increase in cell iodine uptake.

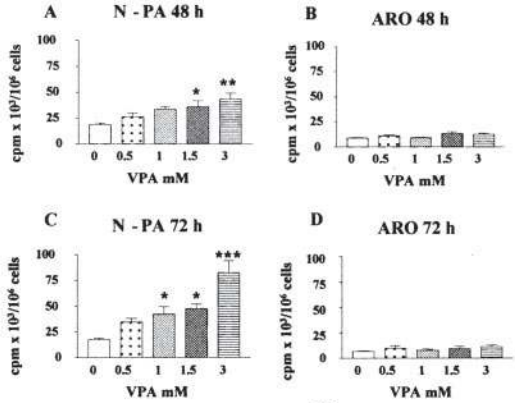

Fig. 4. Effect of valproic acid on ^{125}I accumulation in N-PA and ARO cells after 48 (panel A and B) and 72 (panel C and D) hours treatment. ^{125}I uptake was assayed for 30 min at 37°C. Results are expressed as means ± SEM, n = 3. Treated cells *vs* control (0): $P<0.05$ *; $P< 0.01$ **; $P< 0.001$ ***.

Effect of VPA on membrane expression of NIS

Both N-PA and ARO cells were negative for NIS immunochemistry in basal conditions (Fig. 5, A and D). VPA treatment caused NIS to appear on N-PA membranes (B), while no immunostaining was detectable on ARO membranes (E). In permeabilized N-PA and ARO cells we observed the presence of cytoplasmatic NIS (C, F).

Discussion

This study for the first time shows VPA to be a differentiating agent in thyroid cancer cells, since it induces expression of the NIS gene and increases iodide uptake. It also shows NIS protein to be properly localized at the membrane level in N-PA cells after VPA treatment.

The possibility to achieve expression and modulation of NIS in thyroid tumor cells in order to reverse the loss of iodide uptake observed in dedifferentiated thyroid tumors has long been explored.

Among many substances tested, retinoic acid, which has growth-inhibiting and differentiating properties, has been considered quite promising. The model for retinoic acid therapy is acute promyelocytic leukemia, where up to 90% remission can be achieved (13).

Unfortunately, although an increase in NIS mRNA has been described *in vitro* in some follicular carcinoma cell lines (FTC-133 and FTC-238) treated with retinoic acid, iodide transport has not successfully been reactivated (14).

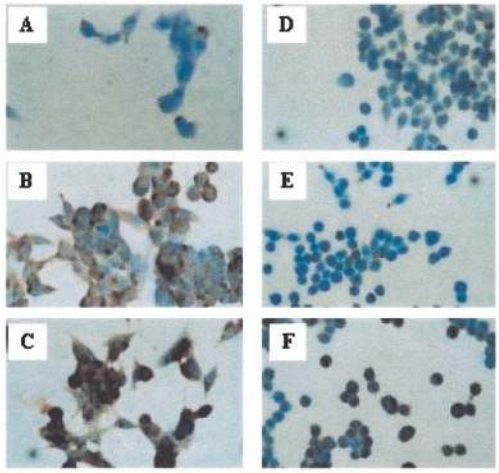

Figure 5- NIS IC of N-PA and ARO cells. N-PA cells in basal conditions (A); N-PA cells treated with 3 mM VPA (B); permeabilized N-PA cells treated with VPA (C). ARO cells in basal conditions (D); ARO cells treated with 3 mM VPA (E); permeabilized ARO cells treated with VPA. Magnification, A-F, x250.

Moreover, a clinical study using retinoic acid in recurrent/metastatized thyroid cancer with no/insufficient iodide uptake, reported an improvement in only 20% of patients and concluded that the therapeutic effect of retinoic acid is certainly less than previously reported (15).

The failure of retinoic acid to reactivate iodide uptake may be attributed to the alteration in NIS trafficking (16). Here we show that in N-PA cells VPA promotes the expression of NIS mRNA, the correct localization of NIS on membranes and, therefore, the increase of iodide uptake. Another HDAC inhibitor, depsipeptide, has been shown to induce expression of NIS and to induce iodide uptake in poorly differentiated thyroid cancer cells (17). The anticancer potential of HDAC inhibitors (18) stems from their ability to affect several processes that are deregulated in neoplastic cells. Alongside activation of differentiation programs, inhibition of the cell cycle and induction of apoptosis are the key antitumor activities of HDAC inhibitors. Various leukemias and solid tumors express differentiated characteristics and stop proliferating when treated with HDAC inhibitors (19).

Unfortunately, patients treated with depsipeptide, as well as with other HDAC inhibitors, for neoplasms other than thyroid cancer, encountered severe gastrointestinal, cardiac and skin toxicity (20). Conversely, VPA shows mild adverse effects in man, even though serum levels exceed the normal therapeutic range during antiepileptic therapy (9). We observed that the lowest dose of VPA that induces differentiation in N-PA cells is 1 mM. Thus the therapeutic levels of VPA reached in patients treated for epilepsy (0.3-

1 mM) are sufficient to evoke re-differentiating action, and it is known that this dose is clinically safe and has no severe side-effects. This is the first demonstration of VPA as differentiating agent in thyroid cancer cells. Unfortunately, the drug did not induce iodide accumulation in the anaplastic thyroid cancer cell line ARO, although it elicited NIS mRNA expression, due to the unpaired targeting of NIS on the plasma membrane.

In conclusion, VPA, which modulates gene activity during re-differentiation therapy, appears to be a good candidate for carefully designed clinical trials aimed at restoring iodide uptake in selected patients with poorly differentiated thyroid cancer.

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