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Retinoic Acid Enhances Histamine Content and H1 Receptor Expression in Human Neuroblastoma Cell Line Paju

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Abstract. *Background: A human neuroblastoma cell line (Paju) was induced by retinoic acid (RA) to differentiate into neuron-like cells. Materials and Methods: We studied the expression and the possible role of histamine receptors H1 and H2 in retinoic-acid mediated differentiation by semiquantitative RT-PCR. We studied the effect of exogeneously added RA on the morphological change of the human neuroblastoma cell line and the differentiation was followed by vimentine, glial fibrillary acidic protein (GFAP) and neurofilament (NF) immunostaining. We monitored the change of the histidine decarboxylase (HDC) expression and the histamine content during the RA treatment by immunoblot and flow cytometry methods. Results: Our data showed that H1 and H2 histamine receptors are present on Paju cells. Ten nM RA markedly increased the H1 receptor expression of these cells, while the H2 expression was unchanged. Conclusion: In the RA-treated Paju cells, the histamine content increased compared to the untreated cells, suggesting that neuroblastoma-derived histamine is involved in the regulation of RA-induced in vitro differentiation by H1 receptors .*

Neuroblastoma (NB) is one of the most common solid malignancies of early childhood, being derived from the neural crest (1). NB cells retain some features of neural crest progenitors, such as the ability to undergo differentiation in the presence of appropriate signals. For this reason, when treated with retinoic acid (RA), human NB cells have been used as a model to study differentiation along the neuronal lineage. However, as an alternative pathway to neuronal differentiation, a "flat, epithelioid or substrate-adherent" phenotype has been consistently described in NB cells "*in vitro*" and "*in vivo*" (2).

Histamine is known to act, at least in part, as a growth factor, as production of this monoamine has been found to accelerate the rate of tissue proliferation during wound repair, embryogenesis and malignant growth. Exogenous histamine has been reported as a growth factor for rat astrocytes and human glioma cell lines (3,4). Histamine is a primary amine synthesised from L-histidine by histidine decarboxylase and affects cells and tissues by binding to H1, H2, H3 or H4 receptors. Vasodilatation, increased vascular permeability and contraction of the smooth muscle are H1 receptor-mediated effects. In the skin, histamine induces vasodilatation, oedema and stimulates axon reflexes to cause the typical wheal and flare response. H2 receptor-mediated activities include gastric acid production, inhibition of T cell cytotoxicity, stimulation of proliferation and leukocyte chemotaxis. H3 type receptors have been described recently in the lung and brain. Histamine depresses sympathetic nerve functions by binding to H3 receptors. The H4 receptor exhibits a very restricted localisation and expression is primarily found in the spleen and thymus, T cells, neutrophils and eosinophils, which suggests an important role for the H4 receptor in the regulation of immune function.

Treatment of neuroblastoma cell lines, including many with amplified N-myc genes, with agents such as retinoic acid (RA), (5-7) phorbol ester, (8,9) dibutyryl c-AMP(10) and interferon-(IFN-)(11,12) "*in vitro*" lead to various levels of morphological and biochemical differentiation linked to growth inhibition. Human and mouse neuroblastoma cells extend neurites and elongate axons following RA exposure. RA accomplishes most of its biological functions through interaction with two classes of nuclear receptors, the RA receptors (RAR) and retinoid X receptors (RXR). Of the

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Key Words: Histamine, histamine receptors, neuroblastoma, tumor, retinoic acid.

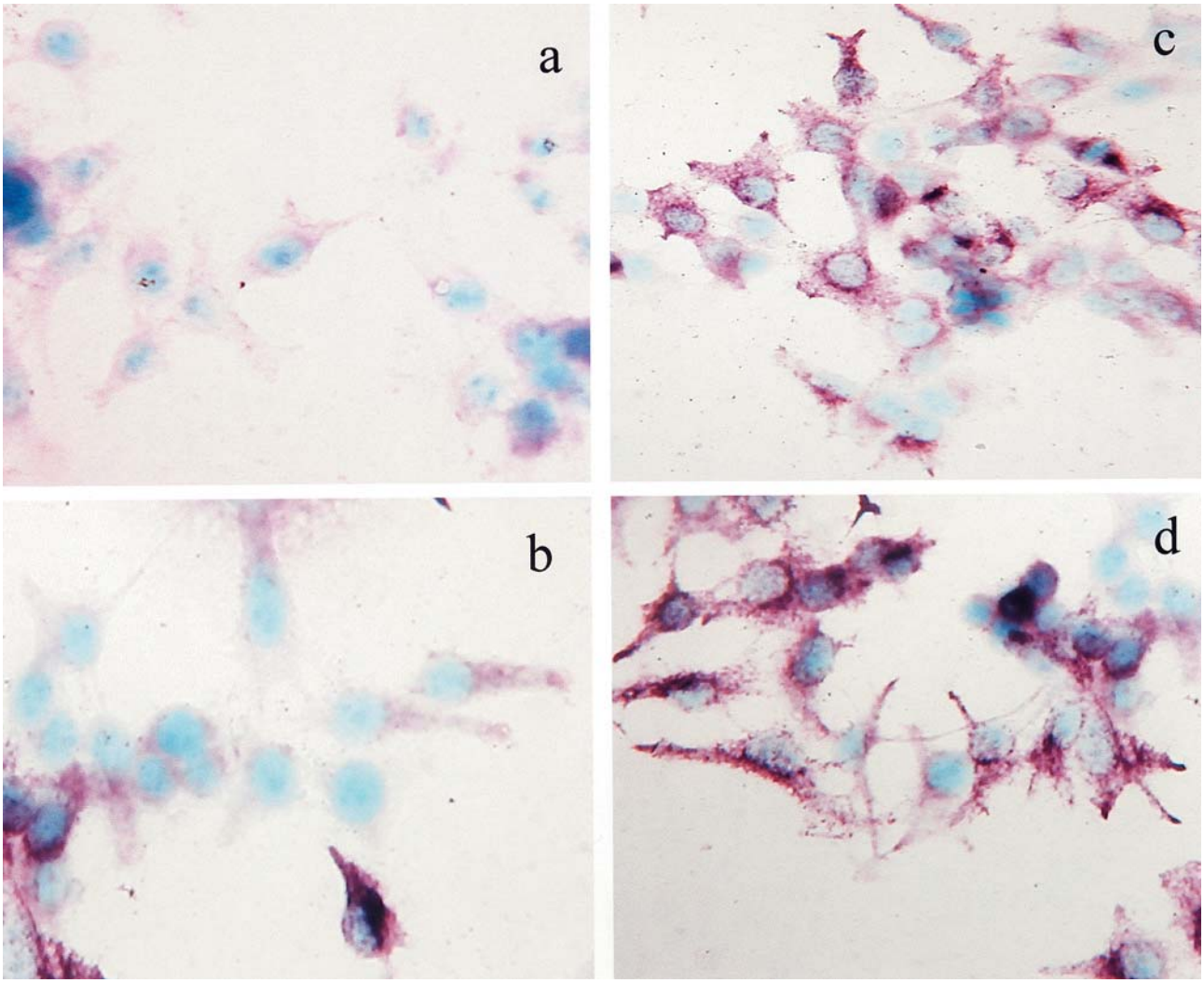

Figure 1. *Morphological differentiation of Paju neuroblastoma cells. Cells were induced to undergo differentiation in the presence of RA (10.0 nM) for 48 h, fixed and stained with anti-HDC to visualise HDC content and study neurite outgrowth and differentiation morphology.*
a, isotypes control; b, untreated control; c, cells treated with 10nM RA for 24 h; d, cells treated with 10nM RA for 48 h. The cells were photographed under an Olympus microscope. Magnification was 40X

naturally occurring retinoids, all-*trans*-retinoic acid (ATRA) binds to both RAR and RXR, whereas 9-*cis*-retinoic acid binds to RXR. The signal is then transduced by the formation of RXR/RAR heterodimers, which bind to RA response elements to activate the transcription of RA-responsive genes.

Paju, a nerve growth factor-independent human neural crest-derived cell line of the primitive neuroectodermal type, undergoes spontaneous differentiation "*in vitro*" (24). Enhanced differentiation, indicated by pronounced neurite outgrowth and neural protein expression, can be induced by Bcl-2 protein overexpression.

The purpose of our study was to establish whether RA induces differentiation on neuroblastoma cells (Paju). Moreover, we investigated whether RA induction acts on histamine synthesis and histamine receptor expression as well.

Materials and Methods

The human neuroblastoma cell line Paju was cultured in RPMI medium supplemented with 10% FCS and antibiotics (Sigma St. Louis, MO, USA). Cells plated the day before the experiment were induced to differentiate in medium containing 1-10 nM all-trans retinoic acid (ATRA, Sigma). Cell number was determined by colorimetric assay, based on the standard MTT (Sigma) staining procedure (13).

Reverse transcription (RT) and polymerase chain reaction (PCR). RT was carried out using Perkin Elmer reverse transcriptase, on a

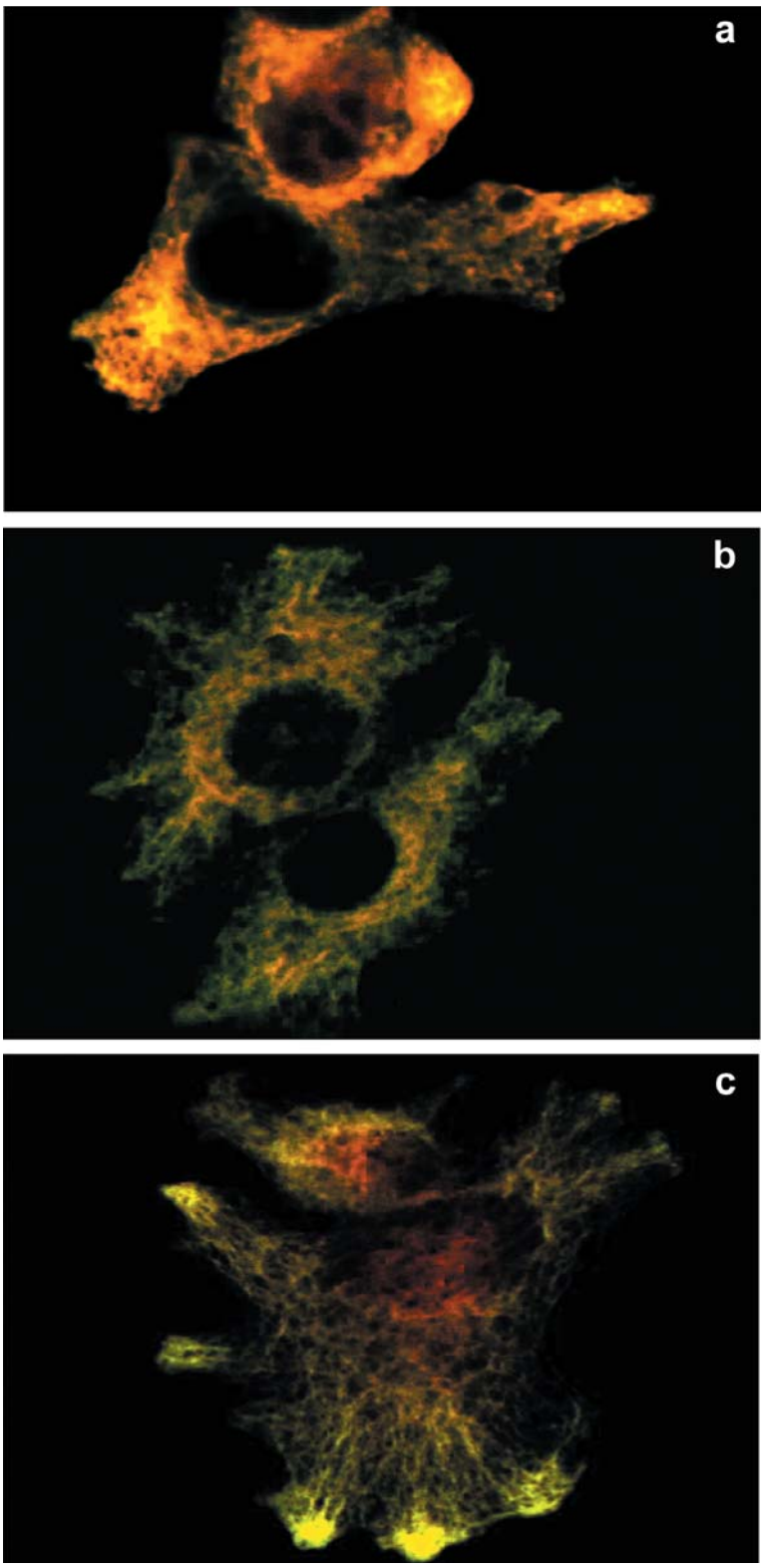

Figure 2. *Immunofluorescence staining of vimentin and GFAP in Paju cells. Double labelling of cells for GFAP using monoclonal anti-glial fibrillary acidic protein (green) and anti-vimentin (red). The untreated control cells (a) or the induced culture by RA for 2 or 3 days (b and c). The cells were fixed and stained with vimentin and GFAP antibodies and photographed under a Biorad confocal microscope. In these cells RA-induced differentiation caused a remarkably reduced amount of vimentin filaments.*

Table I. *Intermediate filament expression in Paju cell line.*

	Untreated	RA-treated
vimentin	++	±
GFAP	+	±
NF (200kDa)	-	-

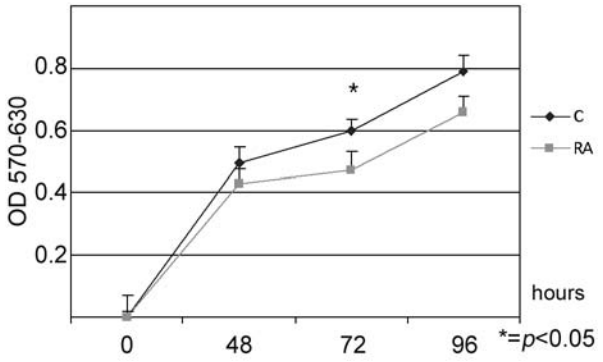

Figure 3. *RA-reduced proliferation of Paju cells. Cells were stimulated for 4 days with 10nM RA and proliferation was measured using colorimetric assay (MTT). Cells were plated out in 0.2ml growth medium in 96-well trays (Nunc) starting at 5x10^3 cells/well. MTT (10µl of 5mg/ml stock) was added in each period of time (after 24, 48, 96 hours incubation) to appropriate wells and the plates were incubated at 37˚C for 3 hours, developed by DMSO and measured by multiwell scanning spectrophotometers (ELISA readers). Results are shown as the mean and standard deviations of 3-4 replicates per point.*

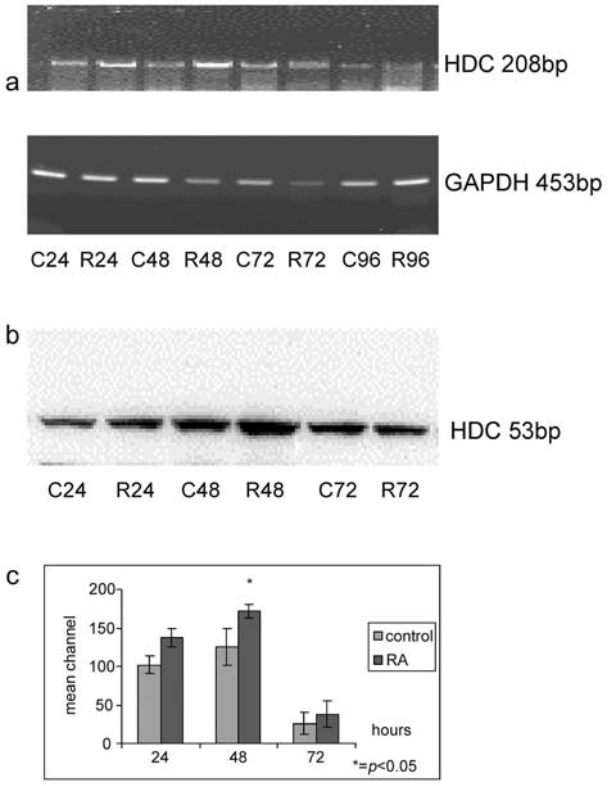

Figure 4. *Effect of retinoic acid (RA 10nM) on the expression of histidine decarboxylase (HDC) Paju cells.*
A. For the kinetics of histidine-decarboxylase induction of total RNA prepared from the treated and untreated cells. RNA was prepared and analysed by RT-PCR as described in Materials and Methods. GAPDH was used as a reference gene.
B. Paju cells were stimulated with 10nM RA (RA) or not (C) for 24-72 hours. Lysates were prepared and equivalent amounts were subjected to SDS-PAGE and Western blot analysis as described in Materials and Methods.
C. Changes of expression of mean fluorescence intensity of HDC during in vitro differentiation, followed by flow cytometry, n=3 (±SD). For details see Materials and Methods

Pharmacia Ataq Gene Controller. The reaction mixture (20 ml) consisted of Mg^{2+} (25 mM) 4 µl, 10x buffer 2 µl, dNTPs (10 mM) 2 µl, RN-ase inhibitor 1 µl (5 units), oligo-dT primer 1 µl, Mu-LV reverse transcriptase 0.5 µl (25 units), DEPC-distilled water , containing 2 µg of total RNA (in 10 µl DEPC-distilled water). RNA samples (2 µg) were reverse transcribed at 42˚C for 30 min, then at 99˚C for 5 min.

For PCR amplification a Promega PCR kit was used. The reaction mixture consisted of 10x buffer 5µl, 1 mM $MgCl_2$, 0.1 mM dNTP-s, 2.25 U Taq polymerase, 25 pmol sense and antisense primers, templates (10 ml cDNA) and distilled water in a 50 ml final volume. The primers were synthesised by Gibco. The PCR reactions were carried out using the following procedures for amplification: 94˚C for 2 min, 30 times (94˚C for 30 sec, 58˚C for 1 min, 72˚C for 1 min), then 72˚C for 7 min for both H1 and H2 receptor. The oligonucleotide primers used for H1 receptor were: 5'-TGG TCA CAG TAG GGC TCA AC sense, 5'-CAA GGT GGG CAG GTA GAA GT antisense; for H2 receptor: 5'-TCG TGT CCT TGG CTA TCA C sense, 5'- CCT TGC TGG TCT CGT TCC T antisense. The HDC PCR was carried out for amplification : 95˚C for 1 min, 30 times (94˚C for 30 sec, 45˚C for 1 min, 72˚C for 1 min), then 72˚C for 5 min, and the human specific sense primer was: 5'-AATCTTCAAGCACATGTC, antisense primer was: 5'-CTGGATAGTGGCCGGGATGA.

The resultant PCR products were separated on 2% agarose gels containing 0.01% ethidium bromide. Samples were run under 100V for 45 min. The sizes of the PCR products 208 bp for HDC, were 497 bp for H1 receptor and 330 bp for the H2 receptor, respectively.

Immunostaining. For intermediate filaments staining, the cells grown on coverslips were fixed in 4% formaldehyde followed by permeabilization in PBS containing 0.1% saponine for 10 min. The preparations were further incubated with anti-vimentine, anti-GFAP or anti-NF monoclonal antibody (Sigma). In several cases we performed double labelling with anti-GFP and with anti-vimentine antibody. The preparations were then washed and placed into solution containing anti-mouse IgG conjugated TRITC, or anti-rabbit antibodies FITC-conjugated goat F(ab)2 immunoglobulin (IgG) (Sigma). The stained cells were examined with the aid of a Bio-Rad MRC 1024 (Bio-Rad, Munich, Germany) confocal laser scanning microscope (CLSM) equipped with

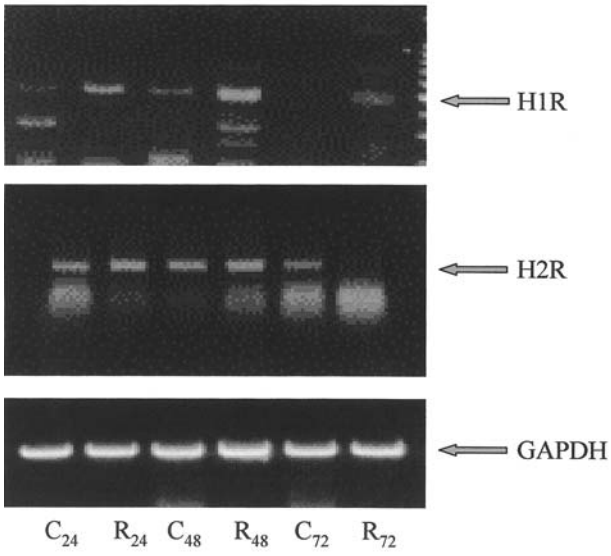

Figure 5. *Retinoic acid (10nM) increases H1 receptor expression at 24 and 48 hours while the H2 receptor expression (RT-PCR) is unchanged in Paju. C: untreated control group, RA: retinoic acid-treated group, GAPDH: glycerine-aldehyde-phosphate-dehydrogenase. Densitometric analysis normalised to the expression of the housekeeping gene glycerine-aldehyde-phosphate-dehydrogenase (not shown) suggested a more than 58% increase in the H1 receptor expression upon incubation with 10nM RA for 48 hours.*

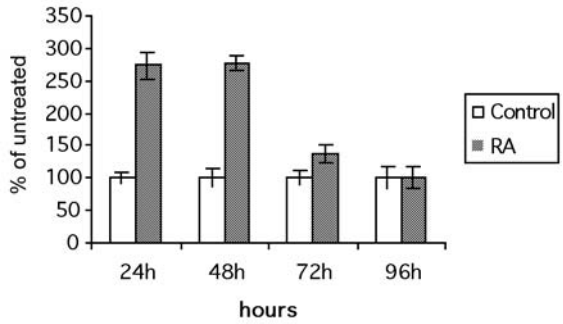

Figure 6. *Changes in amounts of histamine during RA-induced in vitro differentiation of human neuroblastoma cells measured by flow cytometry. The control values were counted as 100% every day and the changes caused by RA were compared to this percentage, n= 3, mean ± SD.*

krypton/argon laser. Control incubation with secondary antibodies alone revealed negligible staining.

For HDC staining, the cells were grown on 4-well chambers in the presence of 10nM RA for 24-72 h and then the slides were fixed with 4% paraformaldehyde for 10 min, washed with PBS, pH 7.4, permeabilized with 0.1% Triton X-100, blocked in PBS with 1% FCS and then incubated with primary anti-HDC antibody (see below) for 1 h. The slides were washed with PBS and incubated with HRPO-labelled secondary antibody, (Promega, Madison, WI, USA) for 1 h. Slides were then washed with PBS, developed with AEC substrate and mounted prior to analysis, using an Olympus BX 50 microscope connected to a digital camera.

Detection of changes in histidine decarboxylase and histamine by flow cytometry. The polyclonal anti-human HDC chicken antibody against epitopes determined by amino-acid 318-325 and the indirect staining method for detecting intracellular HDC was developed by us. The primary polyclonal antibody was produced in chicken to human HDC protein by Promega in collaboration with us (14). After fixation in 4% paraformaldehyde and permeabilization by 0.01% saponine, the cells were labelled by chicken anti-HDC antibodies against 318-325 epitopes and FITC-conjugated anti-chicken secondary antibody. The dilution was 1:1000. Samples were measured within 2 h after labelling. A total of 10,000 cells were measured per sample by FACS Calibur flow cytometer (Becton Dickinson). The analysis was made by CellQuest 3,2 software.

For histamine labelling cells were fixed in 4% freshly-prepared EDAC (carbodiimide), permeabilized by 0.01% saponine and labelled with rabbit-anti-histamine antibody (Sigma) and FITC-conjugated anti-rabbit antibody (Sigma).

Western blot. Cultivated cells were lysed in lysis buffer for protein extraction (containing 10 mM Tris-HCl (tris-hydroxy-amino-methane-HCl) pH=8.0, 10 μg/ml leupeptine, 0.5 mM EGTA 0.5 M, 2% NaF, 1% Triton-X 100, 25 mM PMSF (phenyl-methyl-sulphonyl-fluoride) and 2% Na-orthovanadate). The nuclear fraction was separated by centrifugation (10,000 g, 5 min) and the supernatant was used for determining HDC enzyme by Western immunoblot. For Western analysis an extract of samples were subjected to SDS-PAGE and transferred onto nylon membranes. Each gel lane was loaded with 15 μg of total cytosol protein. The transfers were blocked with Tris-buffered saline containing 0.1% Tween-20 and 1% low-fat milk, probed with 40ng/ml affinity purified $HDC_{318-325}$ antibody, washed, incubated with 0.5 μg/ml HRP conjugate anti-chicken IgY and developed with ECL reagent (Amersham Life Sci, Buckingshire, UK). The identity of the band was controlled by prestained molecular weight standards (full range rainbow marker Amersham Life Sci).

Results

Morphological changes. The phenotypic changes induced by RA typically began after 2 days, were fully manifested after 4 days, but neuritic extension and expression of neuronal markers were consistently absent in RA-treated Paju cells. The cells were flattened , fibroblast-like-shaped, moderately substrate adherent and also frequently had small numbers of finger-like extensions (Figure 1). RA-differentiated cells were extended, rather thick and less branched processes compared to the untreated control cells.

The morphological response of RA-treatment was moderate, but relevant in Paju cells and never led to neurite outgrowth. It may indicate differentiation towards a nonneuronal phenotype. We have not pursued the analysis of this phenotype further.

Marker intermediate filament staining. RA-treated Paju cells showed altered morphology, changing from flat epithelioid

to smaller round or bipolar shapes (Figure 2). Since such morphological changes are suggestive of alterations in intermediate filaments, we have analysed the expression of vimentin, GFAP and neurofilament (200kDa) (Table I).

Immunofluorescent analysis of vimentin showed a redistribution from a cytoplasmic network to a perinuclear accumulation in Paju cell lines. FACS analysis demonstrated that the vimentin content was decreased 20-22% (data not shown). The 200-kD neurofilament protein was not detectable in Paju cells. Vimentin is expressed initially by nearly all neuronal precursors "*in vivo*" and is replaced by neurofilaments shortly after the immature neurons become post-mitotic. Moreover, both vimentin and neurofilaments can be detected transiently within the same neurite, leaving open the possibility that vimentin may play a role in the early stages of neuritogenesis (15).

Effect of RA on proliferation. To evaluate the effect of RA - treatment on neuroblastoma cell growth and viability, the cell lines were treated with 10nM RA for 4 days, during which time the cell number and viability were determined at 0, 48, 72 and 96 hours. Figure 3 showed that RA had only a weak inhibitory effect on Paju cells.

Induction of HDC and H1 receptor expression by RA. In the present study, the mRNA expressions of HDC, H1 and H2 receptors were comparatively analysed in the differentiation of human neuroblastoma cell line induced by retinoic acid (RA).

Paju cells were incubated in the presence of all-trans RA at 10nM for different time periods before RNA was extracted and analysed by RT-PCR. The level of HDC transcript increased after 24 hours of exposure to RA treatment, reached a peak by 48 hours, then returned to that of control cells by 96 hours (Figure 4a).

To determine whether the induction of the HDC gene reflects increased expression of HDC protein, we analysed by FACS and Western blot the abundance of HDC protein content over a 72-hour period following RA stimulation (Figure 4b). RA stimulation caused increase in HDC protein by 24 hours reaching maximal level by 48 hours. To compare the relative quantitative raised amount of HDC we used the Flow cytometric analysis (Figure 4c).

Using a semi-quantitative RT-PCR strategy, we demonstrated a general increase in the mRNA level of HDC and H1 receptor upon differentiation of Paju cells. The semi-quantitative reverse transcriptase (RT)-PCR analysis to confirm the results showed that H1 receptor gene was up-regulated, while H2 receptor was not changed (Figure 5). Thus, our neuroblastoma model system is useful to study the differentiation- and growth-related genes regulated by RA.

HA content. Presence of the active histidine decarboxylase enzyme (HDC) in this cell line is clearly indicated by the change of the histamine production during the RA-induced differentiation. Treatment with 10nM retinoic acid resulted in significant increase (2.74±0.21 or 2.76±01.11-fold, respectively) of intracellular histamine content on days 1-2. Up-regulation of intracellular histamine immunoreactivity by flow cytometry is demonstrated in Figure 6. After the third day the histamine level decreased and HDC expression analysis provided very similar conclusions (Figure 4 a,b,c,).

Discussion

Neuroblastoma cell lines, representing immature, multipotent malignant cells blocked in their differentiation, can be induced to terminal differentiation and permanent cell cycle arrest "*in vitro*" by physiological or nonphysiological agents that apparently re-establish normal growth control (16). Although retinoic acid-induced neurodifferentiation is mediated by interaction with nuclear receptors, the molecular details of how exactly the nuclear message leads to axonal and dendritic outgrowth at the cell membrane level is still not clearly understood. An improved knowledge of the pathways that link differentiation and apoptosis during retinoic acid-induced differentiation would provide a basis for the development of better therapeutic approaches for neuroblastoma treatment.

The present investigation was designed to study the role of HDC, the only enzyme capable of synthesis of histamine in a retinoic acid-induced neurodifferentiation. Cultured human Paju neuroblastoma cells differentiated in the presence of retinoic acid (RA) . The cells acquired long cell processes and the cell growth was partially inhibited. Treatment with RA resulted in an increased HDC expression and activity.

One such agent is the physiological agent RA, which has proven very successful in the treatment of acute promyelocytic leukaemia (17). Based on observations that it can also induce differentiation of neuroblastoma cell lines "*in vitro*" (7-9,16) , RA has been used for several clinical trials in neuroblastoma patients with variable results(18-20). A recent study using high-dose 13-cis RA treatment after myeloablative therapy and autologous bone marrow transplantation showed encouraging results (21,22).

Our results further show that RA reduced vimentin expression. One should, however, point out that the reductions in vimentin expression were moderate and that only 25-40% changes in HDC and H1 receptor expression occurred in response to RA in Paju cells despite clear phenotypic effects.

Our data suggest that human neuroblastoma cells have active HDC and functional histamine receptors which take part in RA-induced differentiation. Thus, our neuroblastoma model system may also be useful to study *in vitro* the differentiation- and growth-related genes regulated by RA (23).

This model cell line can also be used to get new data about the role of histamine in the regulation of cell cycle and differentiation.

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References

1 Dang CV: c-Myc target genes involved in cell growth, apoptosis, and metabolism. Mol Cell Biol *19*: 1-11, 1999.
2 Ciccarone V, Spengler BA, Meyers MB, Biedler JL and Ross RA: Phenotypic diversification in human neuroblastoma cells: expression of distinct neural crest lineages. Cancer Res *49(1)*: 219-25, 1989.
3 Rodriguez J, Moran J, Blanco I and Patel AJ: Effect of histamine on the development of astroglial cells in culture. Neurochem Res *14*: 693, 1989.
4 Van der Ven LTM, Prinsen IM, Jansen GH, Roholl PJM, Defferrari R, Slater R and Der Otten W: Growth of cultured glioma tumour cells can be regulated with histamine and histamine antagonists. Br J Cancer *68*: 475-483, 1993.
5 Haussler M, Sidell N, Kelly M *et al*: Specific high-affinity binding and biologic action of retinoic acid in human neuroblastoma cell lines. Proc Natl Acad Sci USA *80*: 5525-9, 1983.
6 Påhlman S, Ruusala AI, Abrahamsson L *et al*: Retinoic acid-induced differentiation of cultured human neuroblastoma cells: a comparison with phorbolester-induced differentiation. Cell Differ *14*: 135-44, 1984.
7 Sidell N: Retinoic acid-induced growth inhibition and morphologic differentiation of human neuroblastoma cells *in vitro*. J Natl Cancer Inst *68*: 589-96, 1982.
8 Påhlman S, Odelstad L, Larsson E *et al*: Phenotypic changes of human neuroblastoma cells in culture induced by 12-O-tetradecanoyl-phorbol-13-acetate. Int J Cancer *28*: 583-9, 1981.
9 Spinelli W, Sonnenfeld KH and Ishii DN: Effects of phorbol ester tumor promoters and nerve growth factor on neurite outgrowth in cultured human neuroblastoma cells. Cancer Res *42*: 5067-73, 1982.
10 Rupniak HT, Rein G, Powell JF *et al*: Characteristics of a new human neuroblastoma cell line which differentiates in response to cyclic adenosine 3:5-monophosphate. Cancer Res *44*: 2600-7, 1984.
11 Påhlman S and Hedborg F: Development of the neural crest and sympathetic nervous system. Brodeur GM, Sawada T, Tsuchida Y, Voute PA, eds. Neuroblastoma. New York, Elsevier. 2000. pp 9-19.
12 Yeger H, Baumal R, Pawlin G *et al*: Phenotypic and molecular characterisation of inducible human neuroblastoma cell lines. Differentiation *39*: 216-27, 1988.
13 Mosmann T: Rapid colorimetric assay for cellular growth and survival:Application to proliferation and cytotoxicity assays. J Immunol Meth *65*: 55-63, 1983.
14 Haak-Frendscho M, Darvas Zs, Hegyesi H, Karpati S, Hoffman RL, Laszlo V, Bencsath M, Tımar J, Szalai Cs, Fûrész J, Palinger É, Bata-Csorgo Z, Szabad G, Pivarcsi A, Horvath A, Dobozy A and Falus A: Histidine decarboxylase expression in human melanoma J Invest Dermatol *115(3)*: 345-352, 2000.
15 Boyne LJ, Fischer I and Shea TB: Role of vimentin in early stages of neuritogenesis in cultured hippocampal neurons. Int J Dev Neurosci. *14(6)*: 739-48, 1996.
16 Wainwright LJ, Lasorella A and Iavarone A: Distinct mechanisms of cell cycle arrest control the decision between differentiation and senescence in human neuroblastoma cells. Proc Natl Acad Sci USA *98(16)*: 9396-400, 2001.
17 Miller W Jr: Differentiation therapy of acute promyelocytic leukemia: clinical and molecular features [see comments]. Cancer Invest *14*: 142-50, 1996.
18 Finklestein JZ, Krailo MD, Lenarsky C *et al*: 13-cis-retinoic acid (NSC 122758) in the treatment of children with metastatic neuroblastoma unresponsive to conventional chemotherapy: report from the Children's Cancer Study Group. Med Pediatr Oncol *20*: 307-11, 1992.
19 Khan AA, Villablanca JG, Reynolds CP *et al*: Pharmacokinetic studies of 13-cis-retinoic acid in pediatric patients with neuroblastoma following bone marrow transplantation. Cancer Chemother Pharmacol *39*: 34-41, 1996.
20 Smith MA, Adamson PC, Balis FM *et al*: Phase I and pharmacokinetic evaluation of all-trans-retinoic acid in pediatric patients with cancer [see comments]. J Clin Oncol *10*: 1666-73, 1992.
21 Villablanca JG, Khan AA, Avramis VI *et al*: Phase I trial of 13-cis-retinoic acid in children with neuroblastoma following bone marrow transplantation. J Clin Oncol *13*: 894-901, 1995.
22 Matthay KK, Villablanca JG and Seeger RC: Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. N Engl J Med *341*: 1165-73, 1999.
23 Ueda K: Detection of the retinoic acid-regulated genes in a RTBM1 neuroblastoma cell line using cDNA microarray. Kurume Med J *48(2)*: 159-64, 2001.

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