Synergistic effect of retinoic acid and dehydroepiandrosterone on differentiation of human neuroblastoma cells

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Abstract Retinoic acid (RA) affects many cell types by either promoting their survival or inducing their differentiation. Dehydroepiandrosterone (DHEA), a precursor for both androgenic and estrogenic steroids and abundantly produced by brain, is known as an inhibitor of cell proliferation. Differentiation of a human neuroblastoma cell line (SK-N-BE) was evaluated measuring growth rate, motility, neurite extension and GAP-43 expression. We report that DHEA enhances the differentiating effect of RA on neuroblastoma cells via a signalling that is not RA receptor-mediated. Instead, we show a differential expression of matrix metalloproteinases: RA enhances the activity of MMP-2, whereas MMP-9 expression is up-regulated by DHEA. The concerted modulation of these proteinases may support the neurite outgrowth observed after co-treatment with the two drugs.

Key words: Retinoic acid; Dehydroepiandrosterone; Neuroblastoma differentiation; Proliferation; Matrix metalloproteinases

1. Introduction

Retinoic acid (RA) and related vitamin A derivatives exert profound effects on cell proliferation, differentiation, and morphogenesis [1–2]. They are considered important promoters of embryonic development and tissue differentiation maintenance [3]. Retinoids act via two families of nuclear receptors: RA receptors (RARs) and retinoid X receptors (RXRs), members of the steroid/thyroid/retinoid superfamily of inducible transcription factors. Both active as homo- or hetero-dimers, the three subtypes of RARs and RXRs, α, β and γ, differ in temporal and spatial distribution and in ligand affinity [4,5]. RAR β plays a critical role in mediating the growth-inhibitory effect of retinoids in many different types of cancer cells [6,7]. A similar role is exerted by RAR α, whose expression is elicited by estrogens in breast cancer cells [8]. RXRs can heterodimerize also with other nuclear factors of the steroid family, therefore representing a critical checkpoint of several intracellular crosstalks [9,10].

With the aim of investigating patterns of co-operation leading to neuronal cell differentiation, we have focused our attention on steroids whose activity can promote differentiation. Dehydroepiandrosterone (DHEA) and its sulfate ester (DHEAS) are the precursors of both estrogenic and androgenic steroids. DHEA concentrations are particularly high in the brain, and DHEA and related steroids can be synthesized de novo in brain glial cells [11]. Therefore, they are considered 'neurosteroids' and as such it is believed they exert a neuroprotective function, although the molecular mechanisms are not clear [12–14]. Furthermore, it is unknown if all the effects attributed to DHEA are mediated by DHEA or by its metabolites. No nuclear or any other type of receptor system has been described for DHEA or DHEAS. However, recent studies have pointed out alternative mechanisms by which these steroids exert their biological action on nervous system via non-classic steroid hormone receptors [15].

Neurite elongation, the hallmark of neuronal differentiation, results from cytoskeleton reorganization and extracellular matrix (ECM) invasion. Matrix metalloproteinases (MMP) of several kinds are crucial components of the enzyme cascade responsible for ECM remodelling in cell invasion processes, and among them MMPs of 72 kDa (MMP-2 or gelatinase A) and 92 kDa (MMP-9 or gelatinase B) are the most characterized proteinases [16,17]. The differentiating effects of RA also include the control of MMP genes: RA promotes MMP-2 expression levels as well as MMP-9 activity [18,19]. Moreover, DHEAS is able to stimulate the production of gelatinolytic metalloproteinases [20].

Neuroblastoma cell lines are commonly used as in vitro models to study the differentiation and development of neuronal cells. In order to investigate a possible crosstalk of neurosteroids and retinoids in promoting cell differentiation, we used a human neuroblastoma SK-N-BE cell line able to differentiate to a neuronal-like phenotype by producing neurite outgrowth in response to RA [18,21]. DHEA, on the other hand, exerts an anti-proliferative effect on other human neuroblastoma cell lines [22,23].

Evidences provided by this study point out a synergistic effect of RA and DHEA in promoting neuroblastoma cell differentiation, which has been analyzed in terms of growth rate, cellular motility, neurite elongation and expression of a

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Abbreviations: RA, retinoic acid; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; MMP, matrix metalloproteinase; RAR, retinoic acid receptor; RXR, retinoid X receptor; ER, estrogen receptor; GAP-43, growth-associated protein 43

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neuronal differentiation marker, the growth-associated protein 43 (GAP-43). Some aspects of the molecular mechanisms responsible for such a synergy have been investigated.

2. Materials and methods

2.1. Cell culture and proliferation assay

SK-N-BE cells were cultured in DMEM (Gibco, Paisley, UK) without phenol red supplemented with 10% fetal bovine serum, 100 000 U/l penicillin, 100 mg/l streptomycin and 2 mM L-glutamine at 37°C in a humidified 5% CO_2 atmosphere. Cells were treated for the reported time with 10 μM all-*trans* RA (Sigma Chemical, St. Louis, MO), 100 μM DHEA (Fluka, Buchs, Switzerland), RA and DHEA together or solvent alone (ethanol) as control, with periodical replacement of medium. For proliferation studies, cells were seeded in 24-well plastic tissue culture dishes. After nine days of incubation viable cells were stained with 0.1% crystal violet in 20% methanol for 20 min, washed off with water and then solubilized with 10% acetic acid. Optical absorbance was measured at 590 nm using a microplate reader (EL-340, Bio-Tek Instruments, Winooski, VT, USA).

2.2. Movement and neurite growth

For time lapse experiments, cells were seeded in T-25 flasks. After 3 days of treatment the medium was replaced with a HEPES-buffered medium (SGG medium: 114 mM NaCl, 26.1 mM $NaHCO_3$, 5.3 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 1 mM glycine, 30 mM glucose, 0.5 mM sodium pyruvate, 0.001% phenol red). Cell movement was studied over a 12-hour period under a Nikon Diaphot inverted microscope with a 10× phase-contrast objective in an attached, hermetically sealed Plexiglas Nikon NP-2 incubator at 37°C. Cell migration was recorded using a JVC-1CCD video camera. Image analysis was performed with a MicroImage analysis system (Cast Imaging, Venice, Italy) and an IBM-compatible system equipped with a video card (Targa 2000, Truevision, Santa Clara, CA, USA). The sequence of images was analyzed in order to reconstruct the movements, which were then quantified multiplying the length of the path covered by each cell by the distance between the initial and final positions. The first and last frames were compared in order to calculate neurite growth after 12 h of treatment.

2.3. Immunofluorescence analysis

Cells were grown on poly-lysine coated glass coverslips and analyzed as previously described [24] with some modifications. Briefly, at the end of treatments cells were fixed in 3% paraformaldehyde, 4% sucrose in PBS at room temperature for 20 min and washed with 10 mM glycine in PBS. After permeabilization with 0.5% NP-40 in PBS for 5 min, and blocking for 20 min in 1.5% normal goat serum in PBS, cells were treated overnight with a polyclonal antibody against GAP-43 (dil. 1:400; Chemicon International, Temecula, CA, USA). Afterwards cells were washed, incubated with a biotinylated secondary antibody and with fluorescein-avidin D and mounted. Cells were observed under an Olympus fluorescence microscope, and pictures taken with an Olympus DP10 digital photomicrography system.

2.4. Western blot

Immunoblotting of proteins was performed as described previously [25] with the following modifications. Treated cells were harvested by lysis in a sample buffer (100 mM Tris–HCl, pH 6.8, 0.01% bromophenol blue, 2% SDS, 15% glycerol, 10 mM DTT). Equal amounts of proteins (30 μg) were loaded, together with pre-stained full-range molecular weight standards (Amersham, Buckinghamshire, UK) and resolved on 10% SDS–PAGE. Proteins were transferred onto PVD membranes, which were blocked, immunostained with the indicated primary antibodies (all antibodies from Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature, and then with secondary antibody conjugated to horseradish peroxidase. Proteins of interest were detected by ECL method (Perkin Elmer Life Science, Boston, MA, USA).

2.5. Gelatinase zymography

In order to assess the expression of metalloproteinases, gelatin zy-

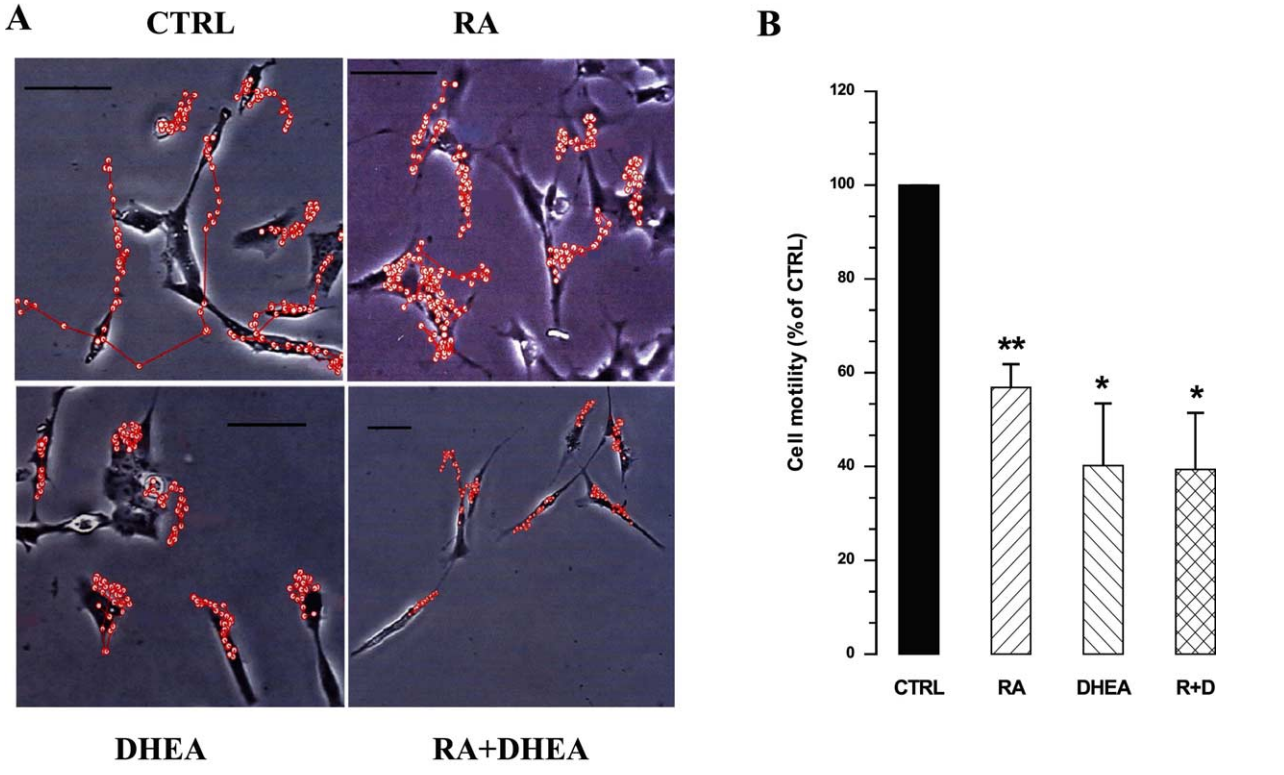

Fig. 1. SK-N-BE cell movements recorded at the third day of treatment with RA and DHEA alone or in combination. Cellular movements were tracked for 12 h and A shows the reconstruction of such movements in a set of experiments representative of a total of three. B: Quantification plotted as a graph, as indicated in Section 2. Values are expressed in comparison to the control and represent the mean ± S.E.M. for $n = 3$. *$P < 0.02$ vs. control; **$P < 0.005$ vs. control. Bar = 100 μm.

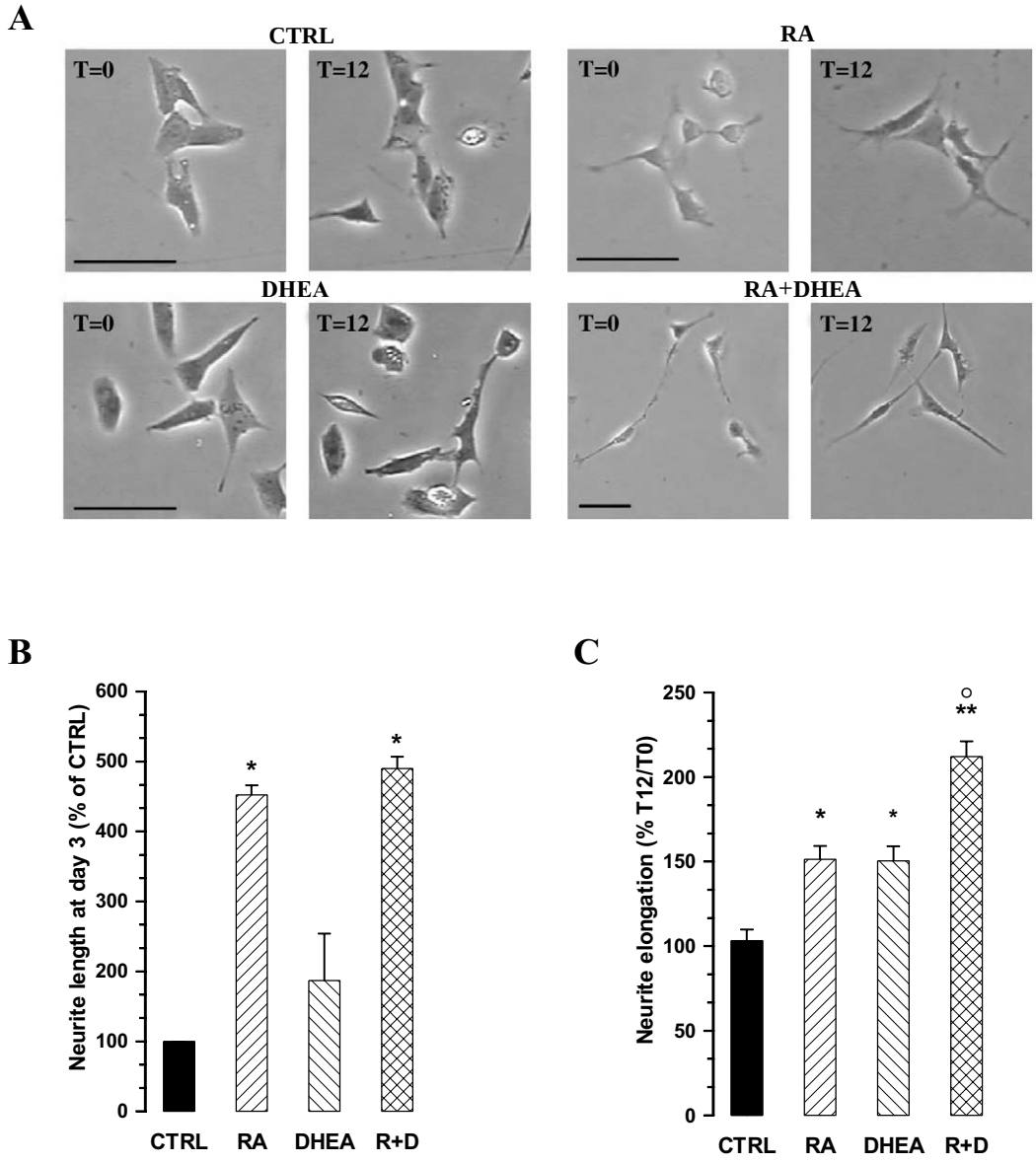

Fig. 2. Neurite growth promoted by RA and DHEA. A: Pictures show a field of control or differentiating cells observed for 12 h as in Fig. 1, and are an example of a frame taken at time zero ($T=0$) and after 12 h ($T=12$), out of three experiments. Using the same frames, initial length of neurites at $T=0$ was measured and plotted on a graph shown in B, whereas in C neurite growth during the 12 h was calculated as ratio between total neurite length measured at $T=12$ and $T=0$. Values plotted are the mean ± S.E.M. for $n=3$. *$P<0.02$ vs. control; **$P<0.002$ vs. control; °$P<0.01$ vs. RA and DHEA alone. Bar = 100 μm.

mogram analysis was performed according to the method of Heussen et al. [26] with some modifications. After 8 days of treatment cells were starved overnight with fresh medium supplemented with 1% FBS, supernatants were collected and cells were lysed in lysis buffer (1% Triton X-100, 50 mM Tris, 300 mM NaCl, pH 7.5). Equal amounts of cell lysate proteins (30 μg) and volumes of conditioned medium proportional to cell numbers (5 μl/10^6 cells) were run under non-reducing conditions in 8% SDS–PAGE containing 0.1% gelatin. After electrophoresis, the gels were washed twice for 1 h each in 2.5% Triton X-100 to remove SDS and then incubated at 37°C in collagenase buffer (50 mM Tris–HCl, 200 mM NaCl, 10 mM $CaCl_2$, pH 7.5) for 16 h or 5 days. After incubation, the gels were stained with Coomassie brilliant blue R-250 and destained in water, allowing the detection of clear areas of digestion in a blue-stained gelatin background.

2.6. Statistical analysis

All data in text and figures are given as means ± S.E.M. Statistical analysis was carried out using the Student's *t*-test for unpaired data.

3. Results

3.1. RA and DHEA inhibit cellular proliferation

Both RA and DHEA are known as growth arrest mediators; we have therefore verified their effect on SK-N-BE cells proliferation. Growth rate of untreated cells or cells treated for 9 days with all-*trans* RA (10 μM), with DHEA (100 μM) and with a combination of all-*trans* RA and DHEA was evaluated by crystal violet staining. Our data confirm the antiproliferative effect of RA and DHEA on neuroblastoma cells, and indicate that growth inhibition is even more evident when the two agents are used in combination (data not shown).

3.2. Effect of RA and DHEA on cellular motility

By time lapse experiments, spontaneous movements of neuroblastoma cells were recorded and evaluated by a software

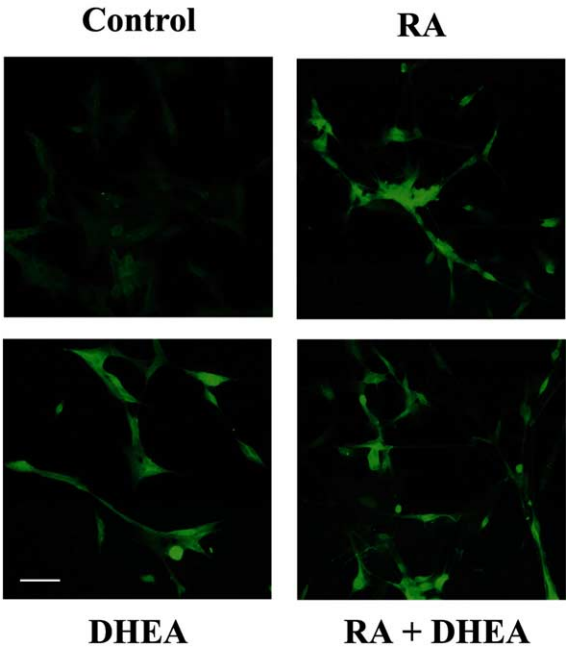

Fig. 3. Increased expression of GAP-43, detected by immunofluorescence after 9 days of treatments. Cells were seeded onto coverslips, treated for 9 days with the two agents alone or in combination, and subsequently stained with anti-GAP-43 antibody. Images were taken from a representative experiment out of three. Bar = 100 μm.

analysis program, as described in Section 2. Quantification of movements discriminates directional movements (evident in control cells) from distances covered around the same spot (detectable in treated cells). As shown in Fig. 1, both RA and DHEA reduce cellular movements, which in differentiating cells become stationary if compared with the random movements of untreated cells.

3.3. RA and DHEA synergistically promote cellular differentiation

The comparison of the neurite elongation in treated and untreated cells shown in Fig. 2A and quantified in Fig. 2B confirms the differentiating effect of RA shown for other neuronal cell lines and reveal a synergy of action between RA and DHEA in promoting neurite extension, already evident at day 3 of treatment (Fig. 2A) and even more at day 9 (Fig. 3). In fact the co-incubation with the two drugs emphasizes the formation of a marked network of neurites. Also, when examined for 12 h, neurite elongation is enhanced by combined treatment with RA and DHEA, as calculated in Fig. 2C.

In agreement with these data, the synergy of RA and DHEA in promoting neuronal-like differentiation is confirmed by the expression of the neuronal differentiation marker GAP-43, which is enhanced by the simultaneous treatment with the two agents, as proved by immunofluorescence experiments shown in Fig. 3.

3.4. Synergistic action of RA and DHEA on neuroblastoma differentiation does not involve alterations in RARs expression

In order to investigate the importance of receptor-mediated signalling promoting differentiation, we evaluated the expression pattern of two RARs, the isoforms β and α, and the expression of one of the modulators of heterodimers formation, RXR β. Moreover, we checked the presence in SK-N-BE cells of one of the candidate receptors for DHEA metabolites, estrogen receptor α (ER α). As shown in Fig. 4, both RAR α and β expression is increased after exposure to RA, as known for other cell lines, but not further enhanced by the co-incubation with DHEA, which has no effect on these receptors, alone or in combination with RA. RXR β expression does not seem to be modulated by either drug. Whatever the differentiating effect of DHEA may be, it does not seem to be mediated by the ER α receptor, because the latter was not detectable on SK-N-BE cells, both in presence or absence of DHEA.

3.5. Modulation of metalloproteinases during differentiation

One of the mechanisms leading to invasion of the extracellular matrix and eventually to cell migration is the enhanced

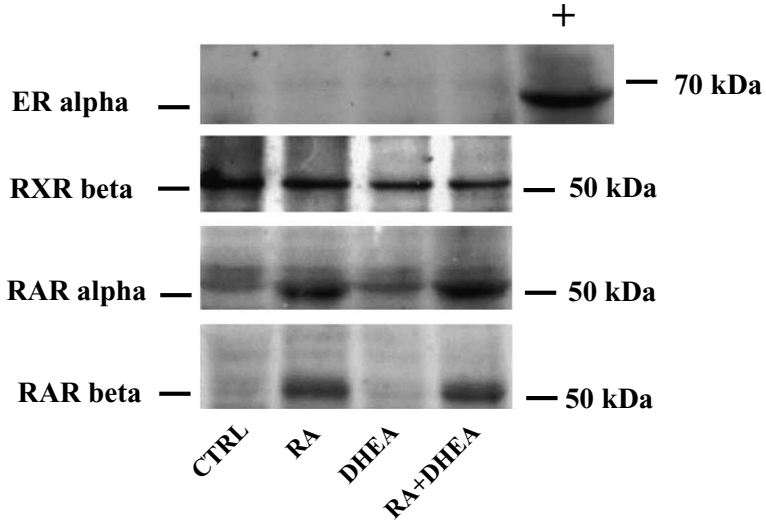

Fig. 4. Western blot analysis of RARs in SK-N-BE cells treated for 9 days with RA and DHEA, alone or in combination. Total cell lysates were immunoblotted and incubated with antibodies against the receptors as marked on the left. A positive control (+: MCF-7 cell extract) was included in the blot incubated with the antibody against ER alpha, in order to check the antibody efficiency and confirm the negative results given by SK-N-BE cell extracts.

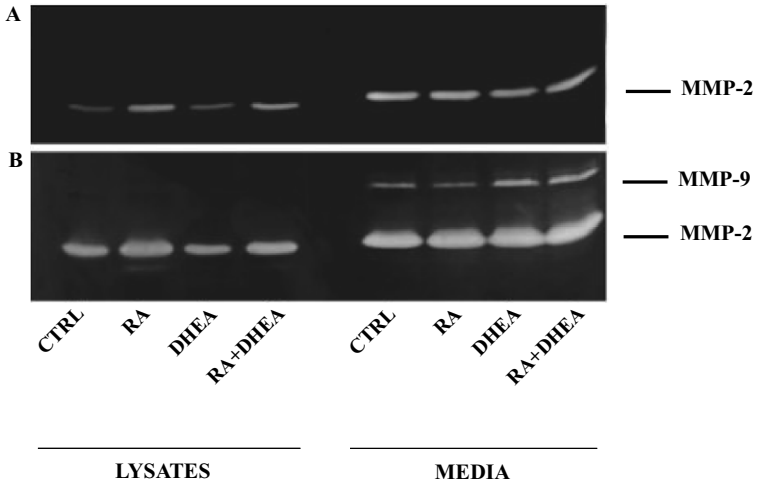

Fig. 5. Effect of RA and DHEA on metalloproteinases activity. Treatment of SK-N-BE cells for 9 days with the two drugs was followed by zymographic analysis of secreted metalloproteinases from conditioned media or cell-associated metalloproteinase activity from cell lysates. Total lysates or conditioned media were electrophoresed and gelatin digestion was carried out for 16 h (A) or 5 days (B), as described in Section 2. Lysis zones corresponding to MMP-9 and MMP-2 are indicated on the right.

production of membrane-bound or secreted metalloproteinases. We therefore analyzed the activity of metalloproteinases produced by untreated cells or after exposure to RA and DHEA. Zymogram studies shown in Fig. 5 reveal the presence of two main metalloproteinases, which due to their molecular weight can be identified as MMP-2 and MMP-9. MMP-2 is detectable both in total cell lysates and conditioned media already after a short incubation (Fig. 5A), and its activity is more evident after a prolonged analysis (Fig. 5B). Only cell-bound MMP-2 exhibits an increase after incubation with RA and RA+DHEA. MMP-9 activity is detectable only after prolonged incubation, suggesting a limited production in SK-N-BE cells, and only in the extracellular medium, indicating that it is totally secreted. Interestingly, MMP-2 is up-regulated during differentiation promoted by RA and not by DHEA, whereas secretion of MMP-9 is enhanced by DHEA and not by RA.

4. Discussion

Differentiation is a process which is in many degrees lost in tumoral cells. All-*trans* RA is particularly effective in inducing the activation of an endogenous differentiation program, however its therapeutic potential is hampered by retinoid resistance associated with many different types of cancer. Little is known of the complex intracellular mechanisms balancing proliferation and differentiation state, and a shift toward the latter could be achieved by combined treatments.

With this in mind, we set out to investigate patterns of enhancement of RA effects. We provide here evidence supporting the potentiating effect of a neurosteroid on RA-induced neuroblastoma cell differentiation. In our study, upon treatment with RA and DHEA, SK-N-BE cells slow down their proliferation rate and develop a differentiated morphology, detectable as neurite sprouting and enhanced expression of a differentiation marker, the protein GAP-43. In agreement with a differentiating process taking place, in the same conditions cellular motility decreases and changes its features, becoming more stationary. Anti-proliferative and differentiating properties of RA and DHEA were already known [27,28], both on neuroblastoma and other cell lines, but we have shown here a reciprocal potentiation of their effect.

Even when grown in vitro, neuroblastoma cells are able to secrete matrix components [29,30], and a boosted ECM remodelling triggered by proteinases could rush the differentiation process. Indeed, in our experimental model increased neurite elongation goes along with a differentially modulated expression of proteinases. In fact RA treatment enhances the expression of the metalloproteinase MMP-2, whereas DHEA exerts its effects increasing the activity of the metalloproteinase MMP-9.

Investigating the molecular mechanisms involved in RA and DHEA synergy, we have found that apparently a differential expression of receptors is not involved. In fact, the RARs most commonly studied in differentiating protocols, RAR α and β, and RXR β, in our experiments give a ligand-induced response (RARs) or a basal expression (RXR) which is not further enhanced by the co-treatment with DHEA. It is not clear whether DHEA acts as such or as precursor of estrogens. Our cellular system does not express the ER α, a well-known player of differentiation events, therefore we can rule out its involvement. Up to now no receptor has ever been isolated for DHEA itself, apart from a reported putative plasma membrane DHEA receptor coupled to G-proteins [31]. Recent experimental evidence indicate that some steroid hormones, besides their well-documented genomic actions, could produce non-genomic rapid effects, such as regulation of calcium transients [15,32]. Interestingly, a differential secretion of MMP-9 upon calcium entry has been reported in keratinocytes and murine carcinoma cells, due to a calcium-responsive element in the promoter region [33], whereas MMP-2 activity seems to be unaffected by calcium signalling [34]. It is therefore reasonable to hypothesize a calcium-dependent transcriptional induction of MMP-9 triggered by DHEA.

Taken together, our data highlight the role of DHEA as enhancer of RA differentiating effect on neuroblastoma cells. Such a synergy might be the result of a concerted regulation at the genomic level (MMPs expression) not involving classi-

cal retinoid receptors but arising from a local modulation of intracellular calcium levels.

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References

[1] Chomienne, C., Fenaux, P. and Degos, L. (1996) Fed. Am. Soc. Exp. Biol. J. 10, 1025–1030.
[2] De Luca, L.M. (1991) Fed. Am. Soc. Exp. Biol. J. 5, 2924–2933.
[3] Ross, S.A., McCaffery, P.J., Drager, U.C. and De Luca, L. (2000) Physiol. Rev. 80, 1021–1054.
[4] Leroy, P., Krust, A., Zelent, A., Mendelsohn, C., Garnier, J.M., Kastner, P., Dierich, A. and Chambon, P. (1991) EMBO J. 10, 59–69.
[5] Zelent, A., Mendelsohn, C., Kastner, P., Drust, A., Garnier, J.M., Ruffenach, F., Leroy, P. and Chambon, P. (1991) EMBO J. 10, 71–81.
[6] Ferrari, N., Pfahl, M. and Levi, G. (1998) Mol. Cell. Biol. 18, 6482–6492.
[7] Liu, Y., Lee, M.O., Wang, H.G., Li, Y., Hashimoto, Y., Klaus, M., Reed, J.C. and Zhang, X.K. (1996) Mol. Cell. Biol. 16, 1138–1149.
[8] Elgort, M.G., Zou, A., Marschke, K.B. and Allegretto, E.A. (1996) Mol. Endocrinol. 10, 477–487.
[9] Kliewer, S.A., Umesono, K., Mangelsdorf, D.J. and Evans, R.M. (1992) Nature 355, 446–449.
[10] Lee, S.K., Choi, H.S., Song, M.R., Lee, M.O. and Lee, J.W. (1998) Mol. Endocrinol. 12, 1184–1192.
[11] Brown, R.C., Cascio, C. and Papadopoulos, V. (2000) J. Neurochem. 74, 847–859.
[12] Lapchak, P.A. and Araujo, D.M. (2001) Int. Rev. Neurobiol. 46, 379–397.
[13] Kaasik, A., Kalda, A., Jaakl, K. and Zharkovsky, A. (2001) Neuroscience 102, 427–432.
[14] Marx, C.E., Jarskog, L.F., Lauder, J.M., Gilmore, J.H., Lieberman, J.A. and Morrow, A.L. (2000) Brain Res. 871, 104–112.
[15] Compagnone, N.A. and Mellon, S.H. (1998) Proc. Natl. Acad. Sci. USA 95, 4678–4683.
[16] Dumas, V., Kanitakis, J., Charvat, S., Euvrard, S., Faure, M. and Claudy, A. (1999) Anticancer Res. 19, 2929–2938.
[17] Bernhard, E.J., Gruber, S.B. and Muschel, R.J. (1994) Proc. Natl. Acad. Sci. USA 91, 4293–4297.
[18] Chambaut-Guerin, A.M., Herigault, S., Rouet-Benzineb, P., Rouher, C. and Lafuma, C. (2000) J. Neurochem. 74, 508–517.
[19] Nie, D., Ishikawa, Y., Yoshimori, T., Wuthier, R.E. and Wu, L.N. (1998) J. Cell Biochem. 68, 90–99.
[20] Sakyo, K., Ito, A. and Mori, Y. (1986) J. Pharmacobiodyn. 9, 276–286.
[21] Melino, G., Draoui, M., Bellincampi, L., Bernassola, F., Bernardini, S., Piacentini, M., Reichert, U. and Cohen, P. (1997) Exp. Cell Res. 235, 55–61.
[22] Gil-ad, I., Shtaif, B., Eshet, R., Maayan, R., Rehavi, M. and Weizman, A. (2001) Isr. Med. Assoc. J. 3, 639–643.
[23] Guarneri, P., Cascio, C., Piccoli, T., Piccoli, F. and Guarneri, R. (2000) J. Neurosci. Res. 60, 656–665.
[24] Neve, R.L., Coopersmith, R., McPhie, D.L., Santeufemio, C., Pratt, K.G., Murphy, C.J. and Lynn, S.D. (1998) J. Neurosci. 18, 7757–7767.
[25] Silvagno, F., Xia, H. and Bredt, D.S. (1996) J. Biol. Chem. 271, 11204–11208.
[26] Heussen, C. and Dowdle, E.B. (1980) Anal. Biochem. 102, 196–202.
[27] McIntosh, M.K., Lea-Furrie, Y.R., Geigerman, C. and Patseavouras, L. (1999) Int. J. Obes. Relat. Metab. 23, 595–602.
[28] Kawai, D., Yahata, N., Nishida, S., Nagai, K. and Mizushima, Y. (1995) Anticancer Res. 15, 427–431.
[29] DeClerck, Y.A. and Lee, C. (1985) J. Natl. Cancer Inst. 75, 431–439.
[30] Linnala, A., Lehto, V.P. and Virtanen, I. (1997) J. Neurosci. Res. 49, 53–63.
[31] Liu, D. and Dillon, J.S. (2002) J. Biol. Chem. 277, 21379–21388.
[32] Kurata, K., Takebayashi, M., Kagaya, A., Morinobu, S. and Yamawaki, S. (2001) Eur. J. Pharmacol. 416, 203–212.
[33] Kobayashi, T., Kishimoto, J., Ge, Y., Jin, W., Hudson, D.L., Ouahes, N., Ehama, R., Shinkai, H. and Burgeson, R.E. (2001) EMBO Rep. 21, 604–608.
[34] Kobayashi, T., Hattori, S., Nagai, Y., Tajima, S. and Nishikawa, T. (1998) Dermatology 197, 1–5.