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Neural Differentiation of Pluripotent Mouse Embryonal Carcinoma Cells by Retinoic Acid: Inhibitory Effect of Serum

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Summary

In both embryonal carcinoma (EC) and embryonic stem (ES) cells, the differentiation pathway entered after treatment with retinoic acid (RA) varies as it is based upon different conditions of culture. This study employs mouse EC cells P19 to investigate the effects of serum on RA-induced neural differentiation occurring in a simplified monolayer culture. Cell morphology and expression of lineage-specific molecular markers document that, while non-neural cell types arise after treatment with RA under serum-containing conditions, in chemically defined serum-free media RA induces massive neural differentiation in concentrations of 10^{-9} M and higher. Moreover, not only neural (*Mash-1*) and neuroectodermal (*Pax-6*), but also endodermal (*GATA-4*, *α-fetoprotein*) genes are expressed at early stages of differentiation driven by RA under serum-free conditions. Furthermore, as determined by the luciferase reporter assay, the presence or absence of the serum does not affect the activity of the retinoic acid response element (RARE). Thus, mouse EC cells are able to produce neural cells upon exposure to RA even without culture in three-dimensional embryoid bodies (EBs). However, in contrast to standard EBs-involving protocol(s), neural differentiation in monolayer only takes place when complex signaling from serum factors is avoided. This simple and efficient strategy is proposed to serve as a basis for neurodifferentiation studies *in vitro*.

Key words

Neural differentiation • Embryonal carcinoma cells • Retinoic acid

Introduction

Retinoic acid (RA), the derivative of retinol, and its signaling pathways, which involve retinoic acid (RAR) and retinoid X (RXR) nuclear receptor-families, play significant roles in the regulation of cell proliferation, differentiation, and apoptosis (Ross *et al.* 2000).

In vitro, RA induces the pluripotent embryonal carcinoma (EC) and embryonic stem (ES) cells to differentiate into various lineages, depending on both the concentration of RA and cell culture conditions

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(Rohwedel *et al.* 1999). In order to be driven to neural lineage, EC/ES cells are normally exposed to RA under non-adherent culture conditions that support the formation of embryoid bodies (EBs). Resulting EBs are then plated on standard tissue culture dishes and further cultured in media without RA (Bain *et al.* 1995, Fraichard *et al.* 1995). The formation of EBs appears to be crucial for neural differentiation of EC/ES cells induced by RA, because the differentiation to endoderm-like cells takes place in monolayer culture in the presence of RA and serum (Jones-Villeneuve *et al.* 1982, Mummery *et al.* 1990, Rochette-Egly and Chambon 2001, Pachernik *et al.* 2002a). However, it was previously documented that growth in serum-free media itself committed EC cells (line C17-S1, clone 1003) to neural differentiation (Darmon *et al.* 1981) and the addition of RA intensified this effect (Tanaka *et al.* 1992). Similarly, cell population rich in neural cells arises from monolayer-grown mouse ES cells that are first allowed to differentiate spontaneously under conditions depleted of the leukemia inhibitory factor (LIF) and feeder cells, and then further cultured in serum-free media (Pachernik *et al.* 2002a). Taken together, these data show that serum-born factors may be strong regulators of RA-directed differentiation in EC/ES cells, which are cultured in monolayer instead of EBs. To address such a possibility in the present study we analyzed the effects of RA on the differentiation of mouse EC P19 cells grown in monolayer culture both with and without serum. Our results document that the neural differentiation of mouse EC P19 cells, driven by serum withdrawal in a monolayer culture, is further accelerated by the addition of RA into media in a dose-dependent manner. However, this "neuropotentiating" effect of RA is completely abolished under serum-containing conditions, thus distinguishing serum-born factors as modulators of RA action on cells of embryonal origin in terms of the specification of differentiation pathways.

Methods

Cell culture and reporter plasmid transfection

EC P19 cells were purchased from the European Collection of Cell Culture, Wiltshire, UK. EC cells were cultured on tissue culture dishes pre-treated for 10 min by 0.1 % aqueous solution of gelatin, in Dulbecco`s modified Eagle`s medium (DMEM) containing 10 % fetal calf serum, 0.05 mM β-mercaptoethanol, 100 i.u./ml penicillin, and 0.1 mg/ml streptomycin, here referred to as standard culture media. Under serum-free conditions, P19 cells were cultured in DMEM/F12 (1:1) media supplemented with the ITS supplement and antibiotics as described above (all GIBCO BRL, Chemos CZ, Prague, Czech Republic). To initiate differentiation, P19 cells ($5x10^3$ per cm^2) were seeded onto the gelatinized tissue culture dishes 24 h before application of experimental conditions. Cells were then treated with various concentration of RA (all-trans retinoic acid, Sigma, Prague, Czech Republic).

Transient transfections of P19 cells were performed by electroporation. Exponentially growing cells ($5x10^6$) were resuspended in standard culture media (400 μl) containing luciferase reporter pRAREβ2-TK-luc plasmid (10 μg, provided by Christopher Glass, University of California, San Diego, La Jolla, CA, USA). The mixtures of cells and DNA were transferred into an electroporation cuvette (4 mm gap) and pulsed (U = 230 V, C = 1010 μF) (Pachernik *et al.* 2002b). Immediately after electroporation, the cell suspensions were transferred into fresh media and plated onto 60 mm gelatinized tissue culture dishes. Twenty-four hours after transfection, culture media was replaced by fresh media containing $5x10^{-7}$ M RA. Thirty-six hours after transfection, the cells were washed with phosphate-buffered saline (PBS) and extracted by 200 μl of lysis buffer for luciferase assay, and luciferase activity was measured according to manufacturer's instructions (Luciferase Assay System, Promega, East Port Praha, Prague, Czech Republic).

Western blot analysis

Neural cell-adhesion molecule (N-CAM, Zaremba *et al.* 1990) and neuron-specific class III β-tubulin isotype (Dráberová *et al.* 1998), both being the markers of neural cells, and endoderm-specific cytokeratin Endo-A (Kanungo *et al.* 2000) were quantified by Western blot analysis. For Western blot analysis, both cultured EC cells and mouse fetal brain tissue (developing neopallium) were processed according to same protocol. Briefly, cells/tissues were washed with PBS and lyzed in sodium dodecyl sulphate (SDS) lysis buffer (50 mM Tris-HCl, pH 7.5, 1 % SDS, 10 % glycerol). Protein concentrations were determined using DC Protein assay kit (Bio-Rad, Bio-Consult, Prague, Czech Republic). Lysates were supplemented with bromphenol blue (0.01 %) and β-mercaptoethanol (1 %), and equal amounts of total protein (10 μg) were subjected

to 10 % SDS-PAGE. After being electrotransferred onto polyvinylidene difluoride membrane (Immobilon-P, Sigma, Prague, Czech Republic), proteins were immunodetected using appropriate primary and secondary antibodies, and visualized by ECL+Plus reagent (Amersham Pharmacia Biotech, Prague, Czech Republic) according to manufacturer's instructions. The following primary antibodies were employed: mouse monoclonal antibody against human N-CAM, which cross-reacts with the mouse homolog (C0678, Sigma), mouse monoclonal antibody against human neuron-specific class III β-tubulin isotype, which cross-reacts with the mouse homolog (TU-20, provided by Pavel Dráber, Institute of Molecular Genetics, Prague, Czech Republic), and rat monoclonal antibody against mouse endoderm-specific cytokeratin Endo-A (TROMA-I, Developmental Studies Hybridoma Bank, University of Iowa). After immunodetection, each membrane was stained by amidoblack to confirm equal protein loading.

Semiquantive reverse transcriptase-PCR

Total RNA was extracted from each cell preparation by the RNeasy Mini Kit (QIAGENE, Bio-Consult, Prague, Czech Republic). Complementary DNA was synthesized according to the manufacturer's instructions for M-MLV reverse transcriptase (GIBCO BRL). Primers and PCR conditions were as follows (primer sequence/annealing temperature/number of cycles/product size):

Pax-6
5`-TGCCCTTCCATCTTTGCTTG-3`, 5`-TCTGCCCG TTCAACATCCTTAG-3`/54 °C/33/178 bp,
Mash-1 (Itoh *et al.* 1997)
5`-CTCGTCCTCTCCGGAACTGATG-3`, 5`-CGACAG GACGCCCGCCTGAAAG-3`/54 °C/30/303 bp,
GATA-4
5`-GAAAACGGAAGCCCAAGAACC-3`,5`-TGCTGT GCCCATAGTGAGATGAC-3`/54 °C/32/186 bp,
α-fetoprotein
5`-ATGTATGCCCCAGCCATTCTGTCC-3`, 5`-GAGA TAAGCCTTCAGGTTTGACGC-3`/54 °C/29/466 bp,
HPRT
5`-CTTGCTGGTGAAAAGGACCTCTC-3`, 5`-CAAAT CAAAAGTCTGGGGACGC-3`/56 °C/24/350 bp.

Before being used for cDNA quantification, the range of linearity was determined for each primer set. PCR products were separated on 1.5 % agarose gels and visualized by ethidium bromide staining (Esner *et al.* 2002).

Results

Differentiation of P19 cells in monolayer serum-free culture

Serum-free culture conditions were previously shown to induce neural differentiation in mouse EC cells, line C17-S1 (Darmon *et al.* 1981). To create the basis for further experimentation, we first tested whether the cells of the mouse EC cell line P19 also acquire a neural phenotype after their adherent culture in media without serum in our experiments. Figure 1a shows that in the absence of serum P19 cells continuously accumulate two different proteins that are typical for neural cells, the neural cell adhesion molecule (N-CAM) and the neuron-specific III β-tubulin isotype, beginning on day 9 of culture. Simultaneously with the accumulation of neural protein markers, the cells adopt neural morphology by first rounding up, creating protrusions, and finally organizing into neurosphere-like colonies with expanding neurites (Fig. 1b).

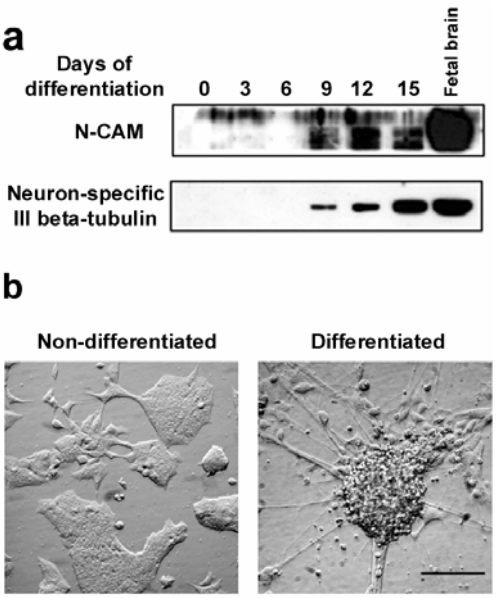

Fig. 1. (**a**) Expression of neural-specific proteins, N-CAM and neuron-specific III β-tubulin, in P19 cells differentiating in chemically defined serum-free media, as determined by Western blot analysis. The lysates were made from P19 cells at various time intervals from the beginning of their differentiation. The lysate made from mouse brain at day 13 of intrauterine development serves as a control. (**b**) Morphology of P19 cells at day 0 (Non-differentiated) and day 12 (Differentiated) of differentiation in chemically defined serum-free media, as observed by Hoffman modulation contrast microscopy. Scale bar – 50 μm. The data are representative of at least three independent replicates.

The effect of retinoic acid on the differentiation of P19 cells in monolayer serum-free culture

P19 cells were exposed to retinoic acid at concentrations of $5x10^{-10}$ M, $5x10^{-9}$ M, $5x10^{-8}$ M, and $5x10^{-7}$ M, respectively, for the initial 2 days of their culture in serum-free media. After another 4 days of differentiation in serum-free media without RA, the expression of both N-CAM and neuron-specific III β-tubulin isotype proteins was analyzed. After a total of six days of experimental culture, the levels of both neural markers were higher in cells treated by RA than in cells differentiated in serum-free media only (Fig. 2). This effect of RA was dose-dependent, being maximally pronounced at a concentration of $5 x10^{-7}$ M.

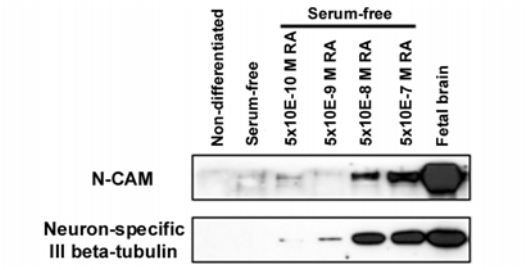

Fig. 2. Expression of neural-specific proteins, N-CAM and neuron-specific III β-tubulin, in P19 cells treated with various concentration of RA. The lysates were made from P19 cells differentiating for 6 days in serum-free media alone (Serum-free) and in the same media supplemented for only the initial 2 days of differentiation with RA at concentrations of $5x10^{-10}$ M, $5 x10^{-9}$ M, $5 x10^{-8}$ M, and $5 x10^{-7}$ M, respectively. The lysates made from non-differentiated P19 cells and from mouse brain at day 13 of intrauterine development were also included. The data are representative of at least three independent replicates.

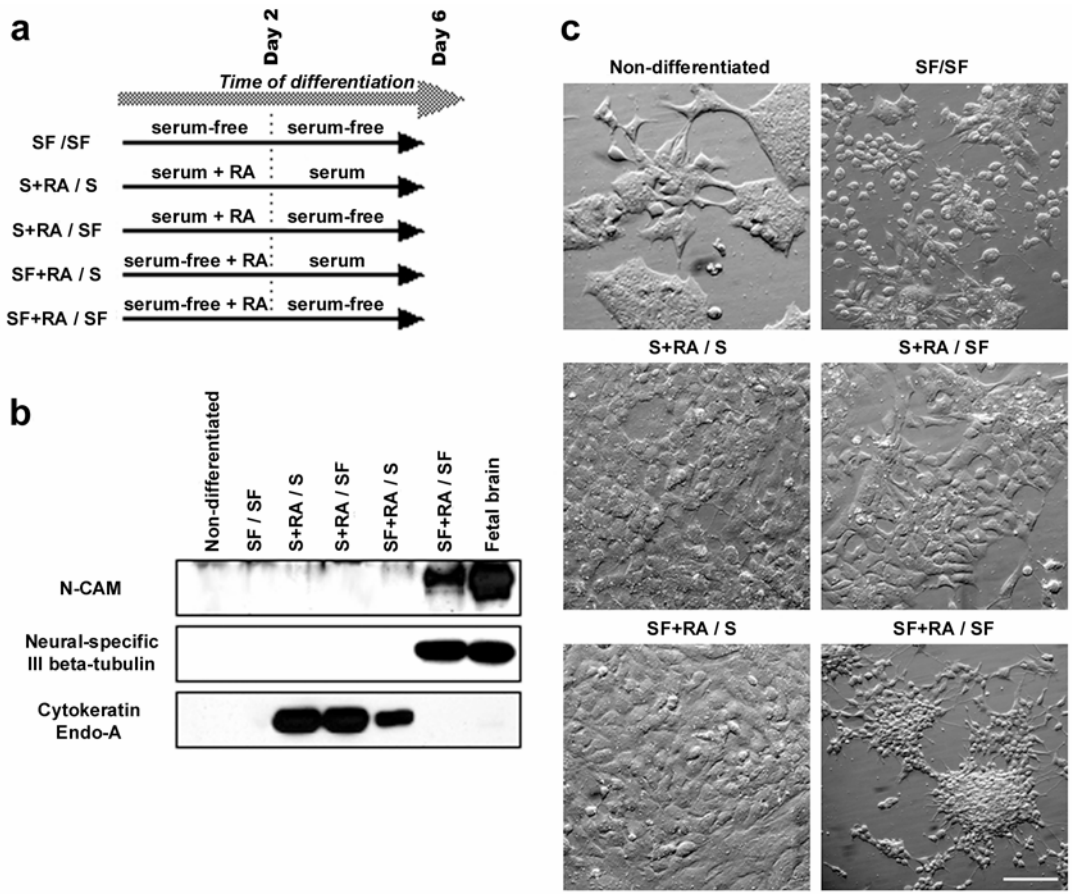

Fig. 3. Phenotype of P19 cells differentiating for a total of six days under various conditions that combine the presence and absence of serum with the treatment by RA. (**a**) Experimental design. (**b**) Expression of neural-specific proteins (N-CAM, neuron-specific III β-tubulin) and endoderm-specific cytokeratin Endo-A in P19 cells differentiating under the conditions specified in panel (**a**), as determined by Western blot analysis. The lysates made from non-differentiated P19 cells and from mouse brain at day 13 of intrauterine development were also included. (**c**) Morphology of P19 cells differentiating under the conditions shown in panel (**a**), as observed by Hoffman modulation contrast microscopy. Non-differentiated P19 cells are also shown. Scale bar - 50 μm. The data shown on panels (**b**) and (**c**) are representative of at least three independent replicates.

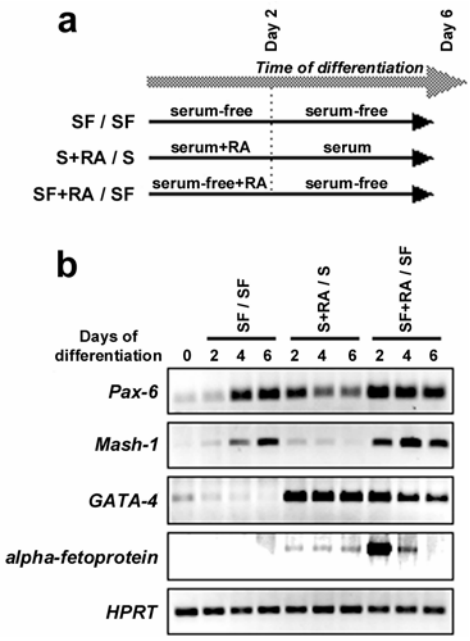

Fig. 4. Expression of lineage-specific genes in P19 cells differentiating under various conditions (schematized in panel (**a**)) for 2, 4, and 6 days, respectively. The levels of mRNAs of neuroectodermal/neural genes *(Pax-6*, *Mash-1*) and endodermal genes (*GATA-4, α-fetoprotein*) shown in panel (**b**) were determined by semi-quantitative RT-PCR. PCR amplification of the HPRT gene served as an internal control for the integrity of cDNA in each sample. The data are representative of at least three independent replicates.

The effect of serum in retinoic acid-induced differentiation of P19 cells

To address the role of serum on RA-induced differentiation of embryonic carcinoma cells, the behavior of neuron-specific III β-tubulin and N-CAM proteins, endoderm-specific cytokeratin Endo-A, and the cell morphology were analyzed. The P19 cells exposed to the following differentiation protocols combining the presence and absence of serum with the presence and absence of $5x10^{-7}$ M RA: (a) 2 days in serum- and RA-containing media followed by 4 days in serum-containing media, (b) 2 days in serum- and RA-containing media followed by 4 days in serum-free media, (c) 2 days in serum-free, RA-containing-media followed by 4 days in serum-containing media, (d) 2 days in serum-free, RA-containing-media followed by 4 days in serum-free media (Fig. 3a). As shown in Figure 3b, among all the tested protocols, only the protocol involving a continuous absence of serum enabled the P19 cells to accumulate both neural markers at day 6 of differentiation. Furthermore, the absence of serum was appropriate for the levels of endoderm-specific Endo-A not to become up-regulated, irrespectively of whether or not the cells were treated by RA. In contrast, Endo-A levels increased when the serum was added to the media both during RA induction and/or after the initial 2-day culture. Microscopical observation revealed that typical P19 cells differentiated by simple continuous culture in serum-free media had a round shape with only some protrusions, while serum-free conditions combined with 2-day exposure to RA (d) invariably resulted in well recognizable neurite outgrowth (Fig. 3c). Most importantly, all culture conditions involving the presence of serum failed to produce any signs of neural differentiation, leaving the cells thoroughly void of a circular shape as well as neurite-like protrusions.

The effect of serum on expression of lineage-specific genes

Endodermal markers appear in monolayer-grown EC cells early after they begin to differentiate in media containing serum and RA (Mummery *et al.* 1990, Rochette-Egly and Chambon 2001). Therefore, we were interested whether the expression of neuroectodermal instead of endodermal early gene markers was typical for P19 cells that were shown to produce cells with neural phenotypes after treatment with RA in the absence of serum. To answer this question, the expression of mRNAs of two neuroectodermal/neural genes (*Pax-6, Mash-1*) and two endodermal genes (*GATA-4, α-fetoprotein*) was determined by semi-quantitative RT-PCR in P19 cells differentiating under various conditions for 2, 4, and 6 days. The conditions were as follows: (a) continuous culture in serum-free media, (b) continuous culture in serum-containing media combined with the exposure to RA for the initial two days, (c) continuous culture in serum-free media combined with the exposure to RA for the initial two days. Taken together, the results summarized in Figure 4, differentiation in serum-free media alone (a) is accompanied by accumulation of only neuroectodermal/neural mRNAs, but the treatment with RA invariably induces the cells to express both neuroectodermal/neural and endodermal mRNAs, irrespective of the presence or absence of serum. Still, while in the presence of serum the levels of *GATA-4* and *α-fetoprotein* mRNAs in RA-treated cells (b) are maintained fairly constant, it is not the case in serum-free culture (c). Correspondingly, the levels of *Pax-6* and *Mash-1* mRNAs are down-regulated with time in cells exposed to RA in serum-containing media, but not in media without serum. Thus, although RA initially

induces the transcription of both neuroectodermal/neural and endodermal genes, the presence or absence of factors contained in serum is decisive for their long-term maintenance.

The effect of the serum on the activity of RARE

The profound modulation effect of serum on RA-induced differentiation observed in this study, led us to ask whether differences in the activity of the RA-directed promotor might underlie this phenomenon. We employed transient transfection of P19 EC cells with the pRAREβ2-TK-Luc reporter plasmid to assay the influence of serum on the activity of the retinoic acid responsive element (RARE). Transfected cells cultured in media with and without serum were treated for 12 h with $5x10^{-7}$ M RA and the activity of RARE was determined in cell lysates by luminometry. As shown in Figure 5, presence and/or absence of serum-born factors did not modulate RA-induced activity of RARE.

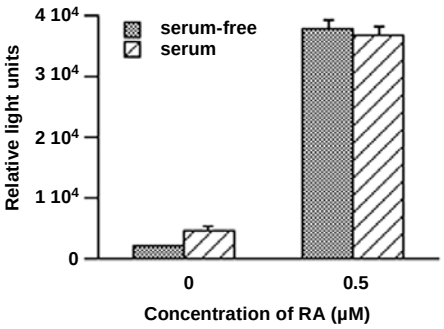

Fig. 5. The effect of serum on the activity of retinoic acid responsive element (RARE). P19 cells transiently expressing pRAREβ2-TK-Luc reporter plasmid were exposed for 12 h to 5 $x10^{-7}$ M RA under the conditions differing by the presence and absence of serum. The activity of RARE element was determined by luminometry in cell lysates. The data are representative of at least three independent replicates. The statistics shown here comes from paralleled assays of one particular experiment.

Discussion

The morphogenic activity of RA and its derivatives made these compounds highly impacted in EC and ES cell research. When these cells are differentiated in the presence of RA, they produce populations rich in specific cell types according to actual culture conditions. In the present study we analyzed the modulating effect of serum on RA-driven differentiation of P19 EC cells under adherent culture conditions.

From the work of Darmon *et al.* (1981) on C17-S1 cell line it became obvious that EC cells follow the neural pathway when differentiated in a simple serum-free monolayer culture. This neural differentiation of EC cells may be further potentiated by an addition of RA (Tanaka *et al.* 1992). Here, we confirmed the general character of such behavior on the widely used P19 EC cell line, and we used this protocol as the basis for neural differentiation of EC cells in one-dimensional culture. We showed that this accelerating effect was clearly dependent on the concentration of RA, only the differences in timing of neural differentiation and not the occurrence of non-neuronal cell types were observed using concentrations of RA in the range of $5x10^{-10}$ to $5x10^{-7}$ M (not shown). In contrast, when RA is applied to EC cells differentiating in three-dimensional embryoid bodies in standard serum-containing media, its concentration strictly determines the developmental pathway, being neurogenic in the case of $5x10^{-8}$ and $5x10^{-7}$ M RA (Bain *et al.* 1994), but myogenic at concentrations of RA that are equal and/or lower than $1x10^{-8}$ M (Edward and McBurney 1983, Rohwedel *et al.* 1999). Together, our data and the data of other authors suggest that it is the lower complexity of culture conditions that allows RA to exert solely its neuro-differentiating potential towards EC cells. This hypothesis is also supported by the effects of adding serum to mouse EC cells differentiating in monolayer under the influence of RA shown here. Our experiments provide a good demonstration that the neurodifferen-tiating activity of RA on EC cells is completely abolished upon the exposure of cells to serum, irrespective of whether such exposure takes place at the very beginning of or later in differentiation. Notably, we also observed the same "deleterious" effect of serum on neurodifferen-tiation in mouse ES cells maintained in monolayer (Pacherník *et al.* 2002a). Although the treatment by RA in the absence of serum causes EC cells to finally develop into neural cells, it still allows for the transitional increase of transcripts of also non-neuronal genes in early stages of differentiation (see *GATA-4* and *α-fetoprotein* in Fig. 4). This fact makes it obvious that certain selection events are involved in RA-driven neurodifferentiation under serum-free conditions. Unfortunately, we can only hypothesize about whether it is (a) the expansion of some cell type that is present in the completely heterogeneous cell population, (b) the molecular change occurring in a rather homogenous population of cells, or (c) the combination of both, which underlies such selection.

Taken together, serum-born factors modulate the effects of RA on EC cells cultured in monolayer, but this modulation does not seem to involve the changes in the pathway transducing the signals from RA onto RARE. Correspondingly, initial step of RA-induced differentiation is, at least in terms of gene expression, rather independent of other factors, to become modulated by serum-born signals only later in the differentiation process. However, the interactions among the signals originating from RA and serum, which inhibited neural differentiation, operate only in EC cells cultured in monolayer and not in three-dimensional embryoid bodies. In other words, at least some differentiating EC cells in embryoid bodies escape from the inhibitory effect of serum and give rise to neural lineage.

Finally, as the formation of embryoid bodies is not in fact required for RA-induced neural differentiation of EC cells, the simple and well defined monolayer-based strategy shown here, which bypasses laborious cell aggregation (McBurney 1993, Bain *et al.* 1994) and/or genome modifying procedures (Boudjelal *et al.* 1997, Sonneveld *et al.* 1999), may be well suited for analyses of the mechanisms that underlie neural differentiation of EC and/or ES cells *in vitro*. Moreover, our results represent a new view on the mechanisms of action a morphogene such as RA and undefined soluble serum-born factors operating in the selection of the cellular fate during mammalian development.

Abbreviations

EBs – embryoid bodies, EC – embryonal carcinoma cells, ES – embryonic stem cells, ITS – insulin, transferrin, selenium, LIF – leukemia inhibitory factor, N-CAM – neural cell-adhesion molecule, RA – retinoic acid, RAR – retinoic acid receptor, RARE – retinoic acid response element, RT-PCR – reverse transcriptase-polymerase chain reaction, RXR – retinoid X receptor.

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Reprint requests

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