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RAPID COMMUNICATION

Retinoic Acid-Induced Neural Differentiation of P19 Embryonal Carcinoma Cells is Potentiated by Leukemia Inhibitory Factor

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Summary

Leukemia inhibitory factor (LIF) is a cytokine that exhibits proliferation, survival and differentiation in a wide range of cell types. Here we show that LIF potentiates retinoic acid-mediated neural induction in pluripotent P19 embryonal carcinoma cells. This activity of LIF was demonstrated by a profounded neural morphology followed by increased expression of neural-specific proteins (N-CAM, III β-tubulin, and GAP-43), up-regulation of early neural lineage-specific gene *Mash-1*, and down-regulation of early endoderm-specific genes *α-fetoprotein* and *GATA-4*. Moreover, LIF also slows growth and increases the level of apoptosis in differentiating cells.

Key words

Neural differentiation • Leukemia inhibitory factor • Retinoic acid • Embryonal carcinoma cells P19

Leukemia inhibitory factor (LIF) is a pleiotropic cytokine that belongs to the interleukin-6 related cytokine family. LIF is synthesized and secreted by various cell types and under *in vitro* conditions it mediates various, in many cases opposite, effects (e.g. proliferation or differentiation). Such effects clearly depend on the cell type and stage of differentiation. *In vitro*, LIF influences several progenitor cell systems and it either promotes the survival and/or expansion of primitive stem cell pools, or enhances the proliferation and differentiation of more committed progenitor cells, usually in combination with other factors (Shellard *et al.* 1996, Metcalf 2003).

LIF could promote self-renewal of mouse embryonic stem (ES) cells while effectively inhibiting their differentiation. Upon LIF withdrawal, ES cells differentiate into various cell types. Similarly, it was determined that LIF can block the differentiation of embryonic stem cell-related P19 embryonal carcinoma (EC) cells which are also broadly used in studies of the differentiation of pluripotent cells (McBurney 1993). LIF inhibits differentiation of P19 cells under several conditions, which lead to endodermal and mesodermal

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cell lineages (Pruitt and Natoli 1992). Importantly, during neurogenesis LIF stimulates the generation, development and survival of neurons from embryonic development to adult (Shellard *et al.* 1996, Cattaneo *et al.* 1999, Auernhammer and Melmed 2000, Shimazaki *et al.* 2001, Metcalf 2003). However, its role in the early induction of neural differentiation is not clearly understood. Therefore in this study we tested the effects of LIF on retinoic acid (RA)-mediated neural induction of pluripotent embryonal cells.

In our previous work we showed that P19 cells might be committed to neural lineages directly by an addition of RA in serum-free media in a monolayer culture (Pacherník *et al.* 2005). Using this simple and chemically well-defined differentiation protocol, we analyzed the effects of LIF on the induction of neural differentiation of P19 cells *in vitro*.

EC P19 cells were treated with RA (0.1 μM; all-trans retinoic acid; Sigma, Prague, Czech Republic), LIF (generally 2.5 ng/ml; Chemicon/ Scintila, Jihlava, Czech Republic) or with a combination of RA and LIF for the first 48 h in a serum-free monolayer culture.

As shown in Figure 1a, LIF alone induced the appearance of protrusions and rounding off of the cell body. Similarly, RA alone induced the formation of protrusions but without extensive rounding off of the cells. However, intensive neurite outgrowth was induced only when the P19 cells were treated with a combination of LIF and RA. The analysis of markers of neural differentiation by Western blot fits well with the changes of P19 cells morphology. Undifferentiated P19 cells did not express a detectable level of neural-specific proteins such as N-CAM, neuron-specific III β-tubulin and GAP-43. When we transferred the P19 cells to a serum-free media, after 96 h of culture the markers of neural differentiation were not yet detected. The presence of LIF in this serum-free media had only marginal effects. The presence of RA induced the expression of neuron-specific III β-tubulin and GAP-43 proteins. No culture conditions were sufficient to induce the expression of N-CAM protein at the time of analysis (Fig. 1b; also in Pacherník *et al.* 2005). In contrast, P19 cells treated with a combination of RA and LIF potentiated the expression of all analysed neural markers (N-CAM, neuron-specific III β-tubulin protein, and GAP-43) (Fig. 1b). We observed no difference on induction of neural differentiation by LIF concentrations between 0.5 and 10 ng/ml of LIF, suggesting that 0.5 ng/ml represents already saturating LIF concentration in our experimental system (data not shown).

In order to obtain a preliminary characterization of the mechanism of the detected effects of LIF on the differentiation of P19 cells we analyzed the expression of genes specific for early lineages, which are induced in P19 cells by RA treatment (neuroectodermal/neural *Pax-6* and *Mash-1*, and endodermal *α-fetoprotein* and *GATA-4*) (Pacherník *et al.* 2005).

Undifferentiated P19 cells did not express a detectable level of mRNA messages, both neuroectodermal (*Pax-6*, *Mash-1*) and endodermal (*α-fetoprotein*, *GATA-4*). The addition of RA to P19 cells results in a continuous accumulation of all four mRNA mentioned above as shown here and previously (Fig 1c; Pacherník *et al.* 2005). Continuous culture in serum-free media down-regulates the level of endoderm-specific messages (*α-fetoprotein*, *GATA-4*) as P19 cells start to be committed to neural lineage. The down-regulation of analysed endodermal genes was well detectable from day 4 (Pacherník *et al.* 2005). An addition of LIF alone did not affect the expression of these genes during the first 48 h. However, when P19 cells were treated with RA and LIF, the accumulation of *Mash-1* message was more potentiated than after the addition of RA only. The accumulation of the *Pax-6* message was slightly lowered compared to treatment with RA alone. On the other hand, the expression of mRNA messages for endodermal lineage specific genes (*α-fetoprotein* and *GATA-4*) decreased both 24 and 48 h after treatment with RA and LIF compared to RA treatment only (Fig. 1c). Our data thus demonstrate for the first time the positive effect of LIF on RA-induced *Mash-1* accumulation and confirm the inhibitory effect of LIF on RA-induced endodermal differentiation, as published previously (Mummery *et al.* 1990, Pruit and Natoli 1992).

The process of cell differentiation is frequently associated with changes in cell growth and the level of apoptosis in differentiating cells. Analysis of P19 cells showed that the presence of RA alone inhibited the growth of P19 cells in serum-free media. The addition of LIF decreased the growth of P19 cells alone and also potentiated the growth-inhibiting effect of RA (Fig. 1d). The analysis of apoptosis shows that when P19 cells grow under standard (optimal) conditions, apoptosis markers such as Lamin B cleavage and DNA fragmentation (DNA ladder) are not detectable.

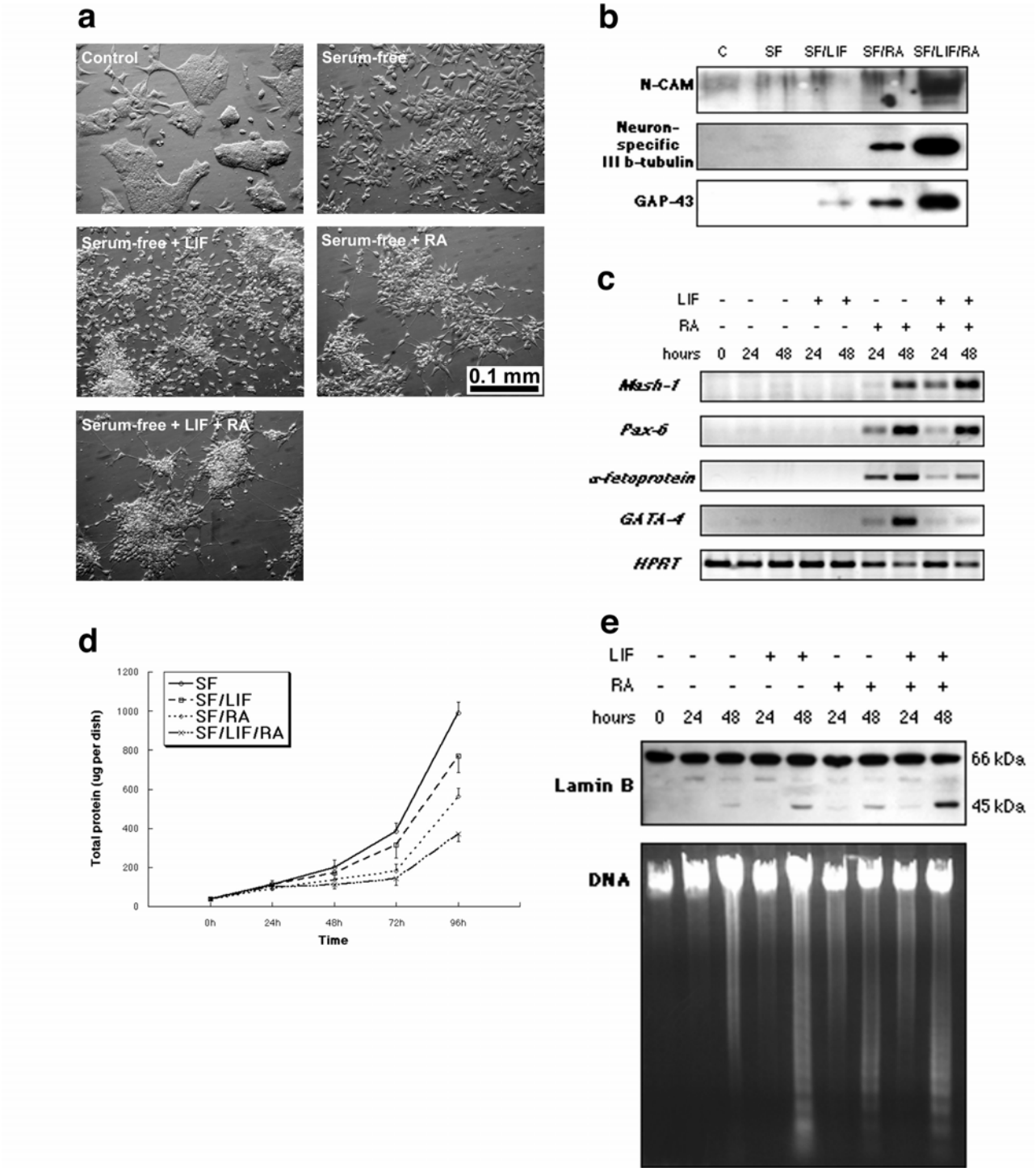

Fig. 1 (**a**) Light micrographs of EC P19 cell morphology under various experimental conditions. Slides photographed with Hoffmann optics 96 h after induction. (**b**) Detection of expression of neural specific proteins as a differentiation marker with Western blotting analysis 96 h after treatment. The following antibodies were used at the indicated dilutions: N-CAM (1:1000, Sigma C0678), Neuron-specific III β-tubulin (1:1000, TU-20 clone, provided by Pavel Draber, IMG, Praque, Czech republic), and GAP-43 (1:1000, Santa Cruz sc-7457). "SF" indicated serum-free media, "C" indicated control, non-differentiated cells. (**c**) RT-PCR assay of expression of pro-neural (*Mash-1* and *Pax-6*) and pro-endodermal (α-*fetoprotein* and *GATA-4*) marker genes in differentiated EC P19 cells 24 and 48 hours after induction. PCR amplification of the *HPRT* gene served as an internal control for the integrity of cDNA in each sample. (**d**) Growth of EC P19 cells under experimental conditions as determined by measuring total cell protein per culture dish. The data showed the average and S.E.M. from three independent assays. (**e**) Induction of apoptosis during differentiation of EC P19 cells 24 and 48 hours after induction. Detection of the cleavage of nuclear envelop protein Lamin B from 66 kDa of full length to 45 kDa fragment with Western blotting assay and DNA fragmentation visualized by agarose gel electrophoresis are shown. All data are from at least three independent experiments. Cells were cultured on gelatinized tissue culture dishes in Dulbecco`s modified Eagle´s medium (DMEM) containing 10 % of fetal calf serum, 0.05 mM β-mercaptoethanol, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin. Under serum-free conditions (SF), P19 cells were cultured in DMEM/F12 (1:1) media supplemented with the ITS supplement (the mixture of insulin, transferrin, and selenium) and antibiotics as described above (all GIBCO BRL, Chemos CZ, Prague, Czech Republic; Pacherník *et al.* 2005). To initiate differentiation, P19 cells ($5x10^3$ per cm^2) were seeded into the gelatinized tissue culture dishes 24 hours before induction of differentiation. Cells were then washed with PBS, overlaid with serum-free media and treated with LIF (2.5 ng/ml) and/or RA (0.1 μM) and their combination. Analysis of differentiation and apoptosis were performed as described previously (Pachernik *et al.* 2002, Pacherník *et al.* 2005; Bryja *et al.* 2004).

After transfer of P19 cells to serum-free media the Lamin B cleavage as well as DNA fragmentation can easily be detected 48 h after treatment. The presence of LIF, RA or a combination of RA and LIF, further increased both Lamin B cleavage and DNA fragmentation (Fig. 1e). This LIF induced potentiation of apoptosis is probably connected with the observed inhibition of P19 cell growth under these culture conditions, similarly as the generally known growth inhibition and apoptosis induction effects of RA on many cell lines (Nagy *et al.* 1998).

The LIF also induces apoptosis in a culture of sympathetic neurons (Kotzbauer *et al.* 1994, Savitz and Kessler 2000), but it promotes the survival of a large number of other populations of neurons (Thaler *et al.* 1994, Murphy *et al.* 1997) and other cell types (Shellard *et al.* 1996, Metcalf 2003). However, the mechanisms of the cell-specific effect of LIF on regulation and processing of apoptosis are still unknown and require further studies.

Our results document the possible role of LIF in the induction of early neural phenotype of pluripotent cells, in the present study represented by the P19 EC cell line. The observed regulation of expression of lineage-specific genes *Mash-1, α-fetoprotein* and *GATA-4*, partly precedes the increase of apoptosis and the main changes in cell growth revealed by a comparison of P19 cells treated either with RA alone or with a combination of RA and LIF. That is why we suggest that these effects of LIF may be caused by direct regulation of differentiation towards neural lineage by a LIF-activated signal transduction pathway rather than by LIF-mediated selection of neural-committed cells from a heterogeneous pool.

Tropepe *et al.* (2001) showed that LIF might act in a permissive manner to enable ES cells to adopt a neural stem cell fate. However, in agreement with these results we did not observe any direct pro-neural fate inducing activity of LIF. Here we may give a preliminary prediction that LIF modulates the cell state in such a manner that makes stem cells more responsive to neural fate-inducing activity. The potential mechanisms of the observed effects are currently under investigation. In summary, the data presented in this study demonstrate that LIF may enhance neural differentiation of embryonic pluripotent cells in combination with some neural inducers such as retinoic acid *in vitro*.

A similar role of LIF in neural differentiation may also be expected during *in vivo* embryogenesis. Transcripts encoding LIF were first detected at the morula stage, and LIF-R and gp130 mRNA was apparent by the time of the blastocyst stage. This early expression of mRNA encoding for a diffusible form of LIF was localized in the trophectoderm. In contrast to the trophectoderm expression of LIF, LIF-R transcripts were localized to the ICM and were undetectable in the trophectoderm, similar to gp130 mRNA (Murray *et al.* 1990, Nichols *et al.* 1996). Furthermore, the uterine endometrium expresses high levels of LIF mRNA immediately prior to blastocyst implantation. The possibility that LIF expressed in the endometrium is translocated into the inner cell mass of the embryo *via* the polar trophectoderm was also suggested (Bhatt *et al.* 1991). Thus the LIF is present in the early embryo when neuroectodermal lineage is formed, and may cooperate with other neurodifferentiation-inducing factors in neurogenic regions (Pruitt 1992, Pruitt and Natoli 1992). One such factor may also be retinoic acid, which we used in our study and which is first secreted by trophectoderm cells in the blastocyst stage (Parrow *et al.* 1998) and at later stages of development during the formation of the primitive streak and neural plate (Niederreither *et al.* 1997, Ulven *et al.* 2000).

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