Original Article

Neural Differentiation Potentiated by the Leukaemia Inhibitory Factor through STAT3 Signalling in Mouse Embryonal Carcinoma Cells

(neural differentiation / leukaemia inhibitory factor / *STAT3* / embryonal carcinoma cells P19)

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Abstract. LIF is a cytokine playing a key role in the regulation of self-renewal and maintenance of undifferentiated state in mouse ES cells. The response of pluripotent cells to LIF is mediated mainly by the STAT3 and ERK signalling pathways. Recently, we have shown that LIF potentiated retinoic acid-induced neural differentiation of pluripotent mouse embryonal carcinoma P19 cells. Here we demonstrate that pro-neural effects of LIF and partially also of retinoic acid are abolished by inhibition of the JAK2->STAT3 signalling pathway. In contrast, inhibition of the MEK1->ERK signalling pathway does not exhibit any effect. These results suggest that in neurogenic regions, cooperative action of LIF and other neuro-differentiation-inducing factors, such as retinoic acid, may be mediated by the STAT3 signalling pathway.

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Abbreviations: EC – embryonal carcinoma, ERK – extracellular signal-regulated kinase, ES cells – embryonic stem cells, JAK – Janus kinase, LIF – leukaemia inhibitory factor, MAPK – mitogen-activated protein kinase, MEK – MAP/ERK kinase, NCAM – neural cell adhesion molecule, RA – retinoic acid, STAT – signal transducer and activator of transcription.

Introduction

The leukaemia inhibitory factor (LIF) is a pleiotropic cytokine that belongs to the interleukin-6 (IL-6)-related cytokine family. LIF mediates various, in many cases controversial, effects on cell proliferation and differentiation both *in vivo* and *in vitro*. Such effects clearly depend on the cell type and differentiation stage. *In vitro*, LIF influences several progenitor cell systems by either promoting survival and/or expansion of primitive stem cell pools or enhancing the proliferation and differentiation of more committed progenitor cells (Shellard et al., 1996, Metcalf, 2003). LIF signalling is mediated through the LIF receptor (LIFR), which activates the gp130 receptor via LIFR/gp130 hetero-dimerization (Gearing et al., 1992). The activation of gp130 results in rapid activation of Janus kinases (JAKs) and signalling molecules including the transcription factor STAT3 (signal transducer and activator of transcription 3). Another major signalling pathway downstream of LIFR/gp130 is the mitogen-activated protein kinase (MAPK) cascade, here in particular the Ras -> ERK cascade (Seiler et al., 2001; Heinrich et al., 2003).

LIF promotes self-renewal of mouse embryonic stem (mES) cells while effectively inhibiting their differentiation (Chambers and Smith, 2004). LIF is also suggested to block the differentiation of embryonic stem cell-related mouse embryonal carcinoma P19 cells (further referred to as P19) to mesoderm and endoderm derivatives (Pruitt and Natoli, 1992). Additionally, we have recently described pro-neural differentiation activity of LIF in P19 cells induced toward neural lineage by all-trans retinoic acid (RA) in monolayer culture under serum-free conditions (Pachernik et al., 2005b).

P19 cells cultured in monolayer in serum-free media differentiate to neural lineages spontaneously (default pathway), in a similar way as mES cells (Pachernik et al., 2002, 2005a). This neurogenic pathway may be approximately three times shortened in time by exposure of P19 cells to pleiotropic RA. However, although the treatment by RA in serum-free media induces differentiation of P19 cells to finally develop into neural cells in serum-free media, RA does not prevent the increase of non-neuronal gene transcription (such as *GATA-4* and *α-fetoprotein*) connected with differentiation into primitive endoderm (Pacherník et al., 2005a). Interestingly, LIF suppresses the expression of transcripts characteristic of endoderm differentiation and also increases the expression of the pro-neural *Mash-1* transcript in P19 cells induced to differentiate by RA (Pachernik et al., 2005b). Importantly, not only expression of the pro-neural *Mash-1* transcript is significantly increased but also other parameters of neural differentiation are further potentiated by the treatment with LIF and RA compared to the treatment with RA alone. Thus, although LIF alone does not exhibit pro-neural effects on P19 cell differentiation, RA-induced neural differentiation is both significantly accelerated and more uniform in the presence of LIF (Pachernik et al., 2005b). Unfortunately, the molecular mechanism underlying LIF-mediated potentiation of neural differentiation remains unaddressed.

In this study we employed chemical inhibition of JAK->STAT3 and MEK->ERK activities to unravel the pathway(s) that are responsible for the pro-neural effect of LIF. Our results demonstrate that the pro-neural activity of LIF and also RA-induced neural differentiation are both inhibited by the JAK2 inhibitor AG490. In contrast, the MEK1 inhibitor UO126 does not have any significant effect on RA-induced neural differentiation both with and without potentiation by LIF. Therefore, it may be suggested that JAK2 -> STAT3 plays a crucial role in the induction of neural differentiation.

Material and Methods

Culture and differentiation of P19 cells

Embryonal carcinoma (EC) P19 cells were purchased from the European Collection of Cell Culture, Wiltshire, UK. EC cells were cultured on tissue culture dishes pretreated for 10 min with 0.1% aqueous solution of gelatin, in Dulbecco's modified Eagle's medium (D-MEM) containing 10 % foetal calf serum, 0.05 μM β-mercaptoethanol, 100 i.u./ml penicillin, and 0.1 mg/ml streptomycin. Under serum-free conditions (differentiation experiments), P19 cells were cultured in D-MEM/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 (5 x 10^3 per cm^2) were seeded onto the gelatinized tissue culture dishes in complete serum-containing D-MEM media 24 h before application of experimental conditions. The differentiation of P19 cells was induced by replacing the serum-containing media with serum-free media and addition of components to which the cells would be exposed. The cells were treated with 0.1 μM RA (all-trans retinoic acid - Sigma, Prague, Czech Republic) or 2.5 ng per ml of LIF (Chemicon/Scintila, Jihlava, Czech Republic) and combinations of RA and LIF for the first 48 h in serum-free monolayer culture. Cells were cultivated in standard conditions at 37°C in a humidified atmosphere containing 5% CO_2 (Pachernik et al., 2005a, b).

Western blots

Cells were washed with PBS and lysed in sodium dodecyl sulphate (SDS) - lysis buffer (50 mM Tris-HCl, pH 7.5; 1% SDS; 10% glycerol). Protein concentrations were determined using the DC Protein assay kit (Bio-Rad, Bio-Consult, Prague, Czech Republic). Lysates were supplemented with bromphenol blue (0.01%) and 1% β-mercaptoethanol, and equal amounts of total proteins (10 μg) were subjected to SDS-PAGE. After being electrotransferred onto a polyvinylidene difluoride membrane (Immobilon-P, Sigma), proteins were immuno-detected using appropriate primary and secondary antibodies, and visualized by ECL+Plus reagent (Amersham Pharmacia Biotech, Prague, Czech Republic) according to the manufacturer's instructions. The following primary antibodies were employed: mouse monoclonal antibody against human neuron-specific class III β-tubulin isotype, which cross-reacts with the mouse homologue (TU-20, provided by Pavel Dráber, Institute of Molecular Genetics, Prague, Czech Republic), mouse monoclonal antibody against cat HNK-1/N-CAM, which cross-reacts with the mouse homologue (VC1.1), and antibody against phospho-MAPK/ERK and MAPK/ERK (PhosphoPlus MAPK Antibody Kit, New England Biolabs, Biotech, Prague, Czech Republic). After immunodetection, each membrane was stained by amidoblack to confirm equal protein loading.

Reporter assay

The P19 cells were transfected by electroporation (Pachernik et al., 2005a) with 2 μg of pSV2neo (Promega, EastPort, Prague, Czech Republic) and 10 μg of pAPRE-luc (provided by Dr. Atsushi Miyajima, Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan, Matsui et al., 2002) to analyse STAT3-regulated transactivation activity, or with 10 μg of pRAREβ2--TK-luc (provided by Dr. Christopher Glass, Department of Medicine and Center for Molecular Genetics, University of California, La Jolla, CA) to analyse RA-induced transactivation activity. Transfected cells were then selected in medium containing 400 μg per ml of G418 (GIBCO BRL, Chemos, Prague, Czech Republic), cloned, and screened for LIF and RA responsiveness, respectively.

Results

LIF-induced activation of STAT3 and ERK signalling pathways in P19 cells

The activation of the STAT3 signalling pathway in P19 cells cultured in serum-free media was assayed using P19 cells carrying the luciferase reporter vector containing the STAT3 responsive element (pAPRE-luc). LIF induced an increase in STAT3-dependent luciferase activity in P19 cells carrying a reporter construct. When the cells were pre-treated by the inhibitor of JAK2 (AG490, 2.5, 7.5, and 25 μM) for 30 min, the LIF-induced STAT3-dependent luciferase activity was decreased in a dose-dependent manner (Fig 1a). In the presence of RA, the effect of AG490 on LIF-induced STAT3-dependent luciferase activity was about the same, with only a slight decrease probably caused by the overall inhibitory effects of RA on cell growth (Fig. 1a).

The LIF-induced activity of ERK proteins in P19 cells cultured in serum-free media was analysed using the specific anti-phospho-ERK antibody selectively recognizing the active forms of ERK proteins (p42/p44) phosphorylated on thr202/tyr204. As depicted in Fig. 1b, LIF induced ERK phosphorylation in P19 cells within 15 min. Pre-treatment of P19 cells with the MEK inhibitor UO126 (5 μM) for 30 min strongly abolished the LIF-induced ERK phosphorylation. The changes to the phosphorylation status of ERK were the same irrespectively of the presence or absence of RA.

The concentration of both STAT3 and ERK inhibitors used were tested to effectively inhibit STAT3 and/or ERK signalling pathways without significant toxicity on P19 cells in our experimental system (data not shown).

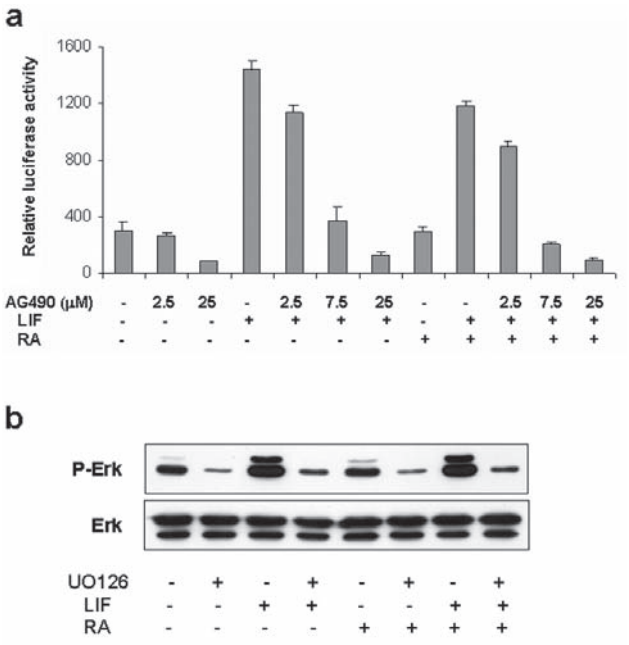

Fig. 1a. LIF-induced STAT3 transactivation is inhibited by the JAK2 inhibitor AG490 in a dose-dependent manner. P19 cells stable-transfected with a reporter vector sensitive to active STAT3 (pAPRE-luc) were pre-treated with or without various concentrations of AG490, as indicated, for 30 min. Further cells were stimulated with LIF or RA and their combinations. Luciferase activity was determined by a luminometer in cell lysates 12 h after cell treatment.
1b. Western blot analysis of LIF-induced ERK activation is inhibited by the MEK inhibitor UO126. P19 cells were pre-treated for 30 min with or without 5 μM UO126 as indicated. Cells were then stimulated for 15 min with LIF or RA and their combination. LIF-induced activity of ERK proteins in P19 cells cultured in serum-free media was tested using specific anti-phospho-ERK antibody selectively recognizing the active form of ERK proteins (p42/p44) phosphorylated on thr202/tyr204. Equal expression of total ERK proteins was confirmed as illustrated.

The effects of inhibition of STAT3 and ERK signalling on LIF-potentiated neural differentiation of P19 cells induced by RA

RA-induced neural differentiation of P19 cells in monolayer culture in serum-free media was documented by the elevation of neural-specific protein markers (neuron-specific III β-tubulin and neural cell adhesion molecule (NCAM)). Consistently with our previously published data (Pachernik et al., 2005a, b), neural differentiation was accelerated by the presence of LIF (Fig. 2.).

To examine the role of the STAT3 and ERK signalling pathways in neural differentiation induced by RA alone and by RA combined with LIF, differentiating P19 cells were treated by JAK2 inhibitor (AG490) and MEK inhibitor (UO126). Specifically, P19 cells grown in serum-free media were pre-treated with AG490 (5 μM) or

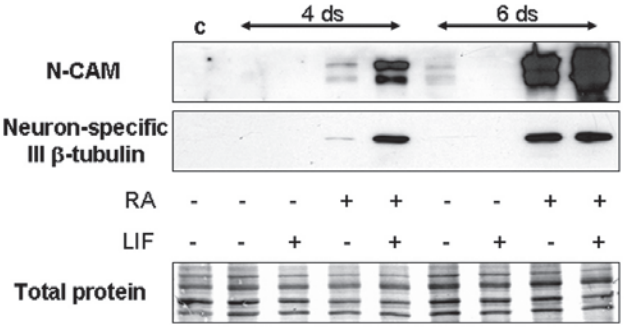

Fig. 2. Expression of neural-specific proteins, NCAM and neuron-specific III β-tubulin by differentiated P19 cells determined by Western blot analysis. The lysate prepared from P19 cells induced to differentiate with RA, LIF, or their combination on days 4 and 6 from the beginning of cell differentiation. "c" represents the lysate from the control, undifferentiated P19 cells. After immunodetection, each membrane was stained by amidoblack to confirm equal protein loading as illustrated.

UO126 (5 μM) for 30 min and then treated with RA and/or RA and LIF for the initial 2 days. After another 2–4 days of differentiation (4 and 6 days of differentiation in total) in serum-free media without any additional substances (inhibitors, RA, and LIF), the state of differentiation was assessed based on the expression of neural-specific protein markers (NCAM and neuron-specific III β-tubulin isotype proteins) and the cell morphology that we have described previously (Pacherník et al., 2002, 2005b). Typically, mouse EC/ES cells differentiating to neural lineages in monolayer culture in serum-free media progressively round up and create protrusions, thus finally generating spherical cell colonies with axonal and dendritic interconnections. Here, on day 4 of differentiation, RA-induced neural phenotype was significantly more pronounced in the presence of LIF, as documented both by higher expression of molecular markers (Figs. 2 and 3a) and better pronounced neural morphology with massively developed cell protrusions (Fig. 3b). Importantly, development of neural characteristics was dramatically affected by inhibiting JAK2 using AG490. Under such conditions the cells failed to upregulate NCAM and neuron-specific III β-tubulin, and also did not adopt a circular shape and/or form neurite-like protrusions (Fig. 3a and 3b). In contrast, inhibition of MEK using UO126 did not affect development of either molecular or morphological aspects of neural differentiation of P19 cells treated by RA and LIF (Fig. 3a and 3b).

It is of note that neural differentiation of P19 cells induced by RA or by combination of RA and LIF for 6 days was inhibited by AG490 to a smaller extent compared to 4 days of differentiation (Fig. 3a). The results suggest that exposure to AG490 for the initial 2 days was not sufficient to produce irreversible block of neural differentiation, as documented by re-established neural molecular signature in cell differentiation for 6 days instead of 4 days (Fig. 3a). This finding further excludes a toxicity of the compound as its mode of action.

The effects of LIF and specific inhibitors of STAT3 and ERK signalling pathways on RA-induced transcription

The effect of the JAK2 inhibitor AG490 on RA-induced differentiation led us to also ask whether differences in the activity of the RA-directed promoter might underlie this phenomenon. To answer this question, we used P19 cells carrying the luciferase reporter vector (pRAREβ2-TK-Luc) containing the retinoic acid-responsive element (RARE). Transfected P19 cells cultured in serum-free media were first exposed or not to the respective inhibitor (AG490 and UO126) for 30 min and then

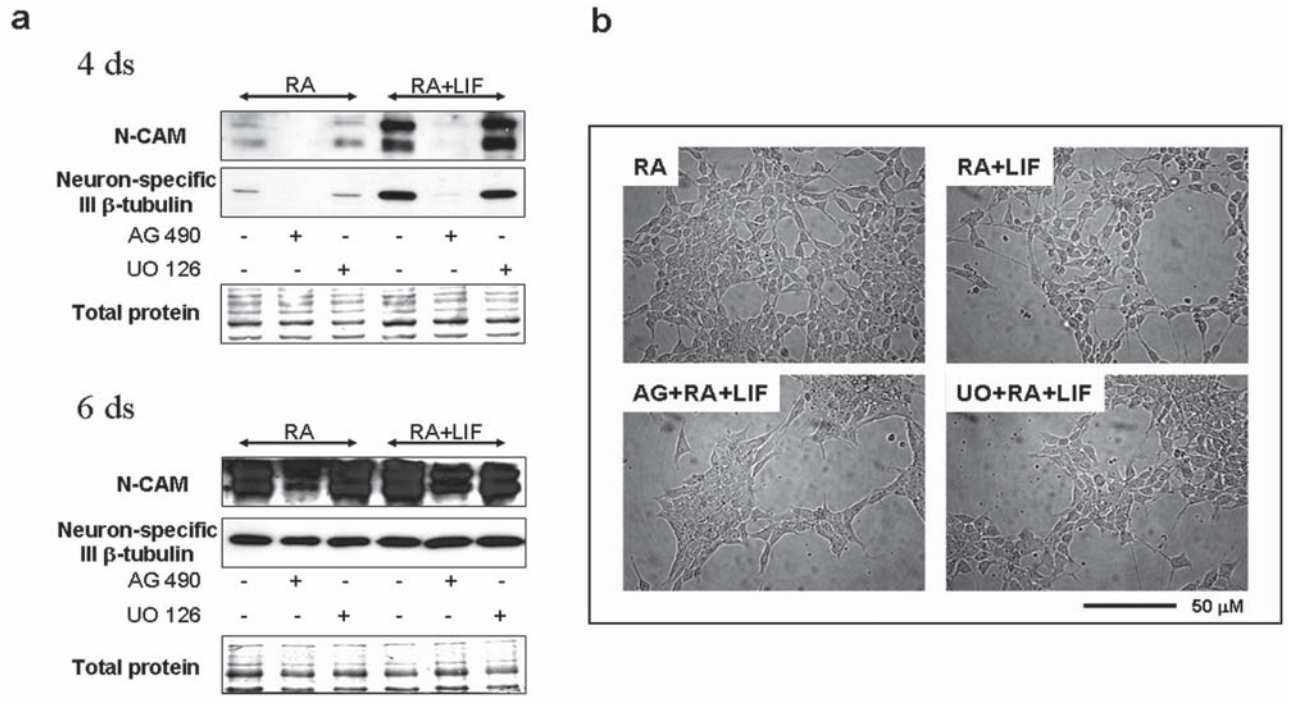

Fig. 3. Effects of JAK2 (AG 490) and MEK (UO 126) inhibitors on LIF-potentiated RA-induced neural differentiation of P19 cells. **a**) Expression of neural-specific proteins, NCAM and neuron-specific III β-tubulin, in P19 cells differentiating in chemically defined serum-free media by RA induction alone and their combination with LIF, and with or without inhibitors of JAK2 (AG 490) and MEK (UO 126) for 4 and 6 days. **b**) Morphology of P19 cells differentiating by RA induction in serum-free media alone (RA) and in the presence of LIF (RA+LIF), LIF and AG 490 (AG+RA+LIF), and LIF and UO 126 (UO+RA+LIF). After immunodetection, each membrane was stained by amidoblack to confirm equal protein loading as illustrated.

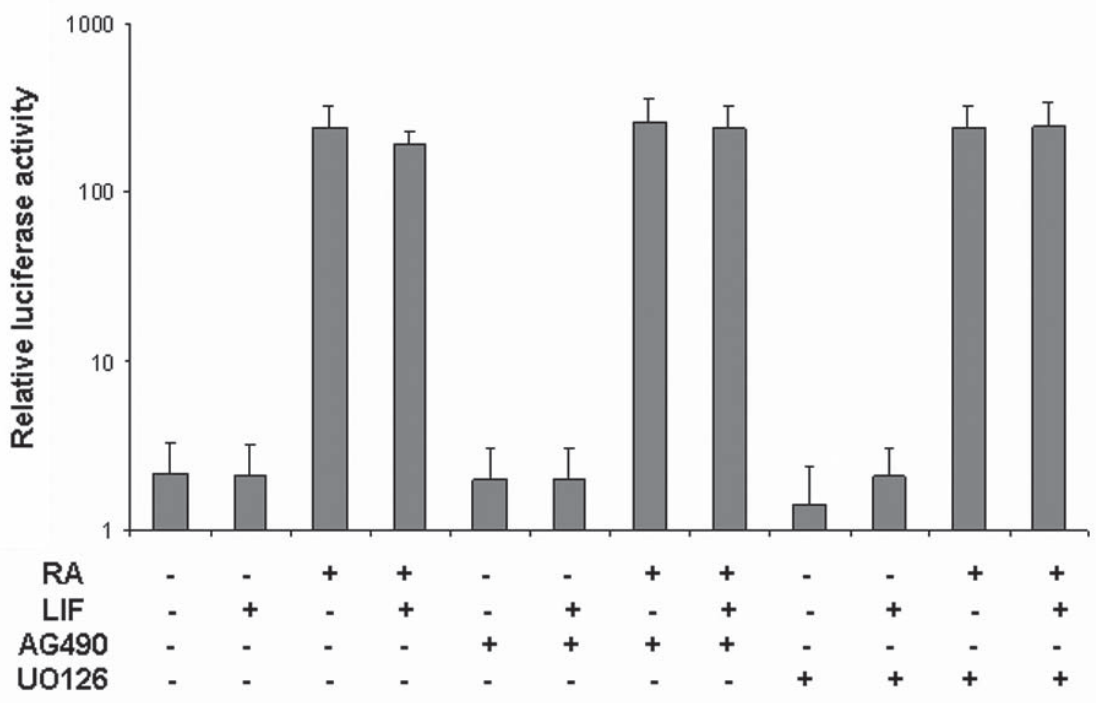

Fig. 4. The effect of JAK2 (AG490) and MEK (UO126) inhibitors, LIF, RA and their combination on the activity of the retinoic acid-responsive element (RARE). P19 cells stable-transfected with pRARE–β2-TK-Luc reporter plasmid were pre-treated for 30 min with or without 5 μM of AG490 or UO126 as indicated. Cells were then stimulated with LIF or RA and their combination. The activity of the RARE element was determined by a luminometer in cell lysates 12 h after treatment.

further cultured for 12 h in the presence of RA, LIF, or a combination of RA and LIF. Under no differentiation-inducing conditions, inhibition of either STAT3 or ERK modified the activity of RARE induced by RA (Fig. 4).

Discussion

Understanding the complex cellular and molecular events that lead to the formation of cell and tissue types making up the higher organism depends at least in part on the identification of molecular players and understanding of mechanism(s) of their action. In this study, we uncovered the new activity of LIF that is mediated via the STAT3 signalling pathway and may play a role during early neurogenesis *in vivo*.

The LIF->STAT3 signalling is the traditional key pathway maintaining the undifferentiated state of mouse embryonal carcinoma and embryonic stem cells (EC/ES). Recently, the self-renewal of mouse ES cells was shown to be completely regulated by the orchestration of LIF and bone morphogenetic protein (BMP) signals. LIF->STAT3 blocks the formation of mesoderm/endoderm derivates and BMP->Smads blocks the formation of the neuroectoderm (Chambers and Smith, 2004). Pruitt and Natoli also observed LIF-mediated inhibition of endodermal and mesodermal differentiation of P19 cells without the inhibition of neuroectodermal differentiation (Pruitt and Natoli, 1992). Similarly, Tropepe et al. showed that LIF might act in a permissive manner to enable ES cells to adopt a neural stem cell fate. However, LIF treatment alone does not induce a direct pro-neural fate of mouse embryonic pluripotent cells confirmed in this study and in the study of Tropepe et al. (Tropepe et al., 2001; Pachernik et al., 2005b). It seems that the pro-neural effect of LIF is dependent on cooperation with other neurodifferentiation-inducing factors such as RA used here. In mouse EC/ES cells, cascades JAK2 -> STAT3 and Ras->MEK -> ERK (MAPK) represent the main signalling pathways that are downstream of LIF. Consistently, LIF also induces rapid activation of ERK and STAT3 signalling pathways in EC P19 cells (Ernst et al., 1996; Schuringa et al., 2002). STAT3 represents an essential molecule in mouse embryogenesis. It was shown that although the LIF -/- mice are viable (Stewart et al., 1992) due to substitution of LIF by other gp130 cytokines present in the early embryo (Seiler et al., 2001), STAT3-deficient embryos develop only into the egg cylinder stage (embryonic day 6) and exhibit rapid degeneration between embryonic days 6.5 and 7.5 (Takeda et al., 1997). In addition, the conventional significance of STAT3 protein during ontogenesis was highlighted by the observation that mice deficient in other types of STAT proteins are viable (Akira, 1999). Thus, other studies should focus on the STAT3 signalling specificity for a particular differentiation pathway.

The STAT3 as well as ERK signalling play a significant role during neurogenesis and both may be induced by ligand-dependent activation of gp130 or other transmembrane receptor kinases (Cattaneo et al., 1999; Mehler and Kessler, 1999). To evaluate the involvement of STAT3 and/or ERK signalling pathways in LIF-induced acceleration of neural differentiation of P19 cells AG490, a specific inhibitor of JAK2 (Schuringa et al., 2002), and UO126, a specific inhibitor of MEK (Jo et al., 2005) were employed in this study. We have found STAT3-dependent transcription activity to be involved in LIF potentiation of RA-mediated neural differentiation in P19 cells in monolayer culture and serum-free conditions. Interestingly, our data also suggest the importance of STAT3 signalling in neural differentiation in general.

In the central nervous system the JAK -> STAT signalling, generally induced by the gp130 cytokine family, regulates proliferation, differentiation, and apoptosis of developing populations of neurons and glia. Especially LIF, mainly through the JAK -> STAT pathway, stimulates the generation, development, and survival of neurons and glia from embryonic development to adult (Shellard et al., 1996; Cattaneo et al., 1999; Mehler and Kessler, 1999; Auernhammer and Melmed, 2000; Shimazaki et al., 2001; Metcalf, 2003). Since P19 cells are postulated to resemble cells of the egg cylinder (Chambers et al., 2003), our results presented here indicate the possible regulatory role of the LIF->STAT3 pathway in such an early step of neurogenesis as a part of the very initial neurogenic signalling. The expression of key molecules mentioned here in early embryo confirms this presumption. RA is synthesized and components of JAK->STAT signalling are expressed in the early embryo already at the blastocyst stage (Parrow et al., 1998; Akira, 1999). At this stage, the inductors and upstream molecules of this pathway such as LIF, high-affinity receptors LIFR, and gp130 are also present (Murray, 1990; Nichols et al., 1996). This is suggestive of a paracrine interaction whereby cytokine production would act to regulate early embryogenesis. Thus, our data demonstrate the possible active role of the LIF/gp130 -> JAK2 -> STAT3 pathway in the earliest steps of neurogenesis.

In conclusion, the results presented here supplement our knowledge about the role of STAT3 in early steps of embryogenesis. STAT3 activity is required for the induction of RA-induced neurogenesis of pluripotent P19 EC cells. The previously observed pro-neural effects of LIF are most probably mediated by LIF-induced STAT3 activity without the involvement of the ERK pathway.

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