A p38 inhibitor allows to dissociate differentiation and apoptotic processes triggered upon LIF withdrawal in mouse embryonic stem cells

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Received 16.4.03; revised 31.7.03; accepted 08.9.03; published online 19.12.03
Edited by ME Peter

Abstract

Mouse embryonic stem cells remain pluripotent when maintained in the presence of leukemia inhibitory factor (LIF). Upon LIF withdrawal, most cells differentiate into various lineages, while some die by apoptosis within 3 days. We have analyzed the activation pattern of the mitogen-activated protein kinase (MAPK) families and characterized the expression profile of selected genes modulated during differentiation or apoptosis. We show that p38 MAPKs are activated first, during the apoptotic crisis, while extracellular-regulated kinases and c-Jun N-terminal kinases are induced after the apoptotic crisis in differentiated cells. However, by using both p38 kinase inhibitors (PD169316 and SB203580) and a p38 α ^{-/-} cell line, we demonstrate that p38 α activation is rather a consequence than a cause of apoptosis. We thus reveal novel properties of PD169316, which induces cell survival without impairing cell differentiation, and identify PD169316-sensitive targets like the *fibroblast growth factor-5*, *Brachyury* and *bcl-2* genes. Finally, we demonstrate that overexpression of the PD169316 – regulated *bcl-2* gene prevents LIF withdrawal – induced cell death.

Cell Death and Differentiation (2004) 11, 331–341. doi:10.1038/sj.cdd.4401337

Published online 19 December 2003

Keywords: apoptosis; caspase-3; ES cells; cytokine; p38; PD169316; SB203580; bcl-2

Abbreviations: Casp3, caspase-3; ES, embryonic stem; ERK, extracellular signal-regulated kinase; EGFP, enhanced green fluorescent protein; fgf, fibroblast growth factor; JNK, c-Jun

N-terminal kinase; ICM, inner cell mass; LIF, leukemia inhibitory factor; MAPK, mitogen-activated protein kinase; PARP, poly-(ADP ribose) polymerase; STAT, signal transducer and activator of transcription; RTQ-PCR, real-time quantitative-polymerase chain reaction.

Introduction

Embryonic stem (ES) cells, which are pluripotent cells derived from the inner cell mass (ICM) of blastocysts, provide a good system to study the molecular determinants involved in early differentiation of the preimplantation embryos. Mouse ES cells, derived from 129 mice strain, are maintained pluripotent *in vitro* in the presence of leukemia inhibitory factor (LIF), a cytokine belonging to a family including interleukin 6, ciliary neurotrophic factor, oncostatin M, cardiotrophin 1 and cardiotrophin-like factor.^{1–5} These cytokines, which transduce their signal through heteromultimeric receptors, share common proliferative, cell survival or differentiative potentials depending on the cell types.^{6–8} While some molecular determinants of ES cell pluripotency are well characterized, like the fibroblast growth factor-4 (FGF-4) and the signal transducer and activator of transcription 3 (STAT3), OCT-4 and NANOG transcription factors, much less is known about the intricate processes of differentiation that start within 36 h upon LIF withdrawal in ES cells.^{9–15}

Cell differentiation and apoptosis are linked and we have shown that upon LIF withdrawal, part of the cells die through apoptosis, concomitantly with an activation of the p38 mitogen-activated protein kinase (MAPK), but independently of the caspase-dependent cleavage of the poly-(ADP ribose) polymerase (PARP)-1 substrate.¹⁶ A LIF-dependent cell survival effect on primordial germ cells has also been described, but the molecular determinants of the antiapoptotic effect of LIF have not been characterized yet.¹⁷

Apoptosis, a multistep genetic program leading to cell death, is involved at early stages of embryogenesis in at least two major processes: proamniotic cavity formation, a morphogenetic process that begins after blastocyst implantation and elimination of cells with damaged DNA.^{18,19} Apoptotic cells are detected in the ICM of the blastocyst in which transforming growth factor α (TGF α) is a critical cell survival factor.²⁰ In addition, the Taube nuss and apoptosis-inducing factor proteins have been characterized as key proteins controlling programmed cell death during early morphogenesis.^{21,22}

Various apoptotic stimuli (UV, osmotic stress as well as particular cytokines) induce independent pathways that include the release of cytochrome c by mitochondria and the activation of different sets of caspases, ultimately leading to DNA degradation and phagocytosis of dead cells.^{23–26} In addition, a subset of MAPKs (belonging to the c-Jun N-terminal Kinase (JNK) and p38 families) and members of

the ribosomal S6 kinase family are instrumental in apoptosis induced by trophic factor withdrawal.^{27–30} Furthermore, JNK1 and JNK2 regulate region-specific pro- or antiapoptotic processes during early brain development.³¹

The p38 MAPK family includes four genes (p38 α , β , γ and δ) encoding proteins induced by osmotic stress, UV and various cytokines involved in inflammatory responses. These kinases are activated through dual phosphorylation at their conserved TGY residues by MKK3, MKK4 and MKK6 kinases, which have different specificities for each p38 protein.³⁰ The study of the role of p38 MAPKs has largely benefitted from the availability of imidazole-based pharmacological inhibitors, like SB203580 and its analogue PD169316, which block the activity of the p38 α and β subtypes by associating within the ATP-binding pocket.^{32,33} However, the specificity of these p38 inhibitors has been challenged and differences in the effects of SB203580 and PD169316 on particular physiological responses have been reported.^{34,35} The p38 kinases have also been involved in differentiation processes and deletion of the p38 α gene leads to early embryonic lethality with various cell lineage deficiencies.³⁶

The *bcl-2* gene family is composed of anti- and proapoptotic members that play a major role in the maintenance of the mitochondria integrity by preventing the apoptosis-induced cytochrome c release.^{37,38} The classical caspase-9/caspase-3 (Casp3) pathway is blocked upon *bcl-2* overexpression, but alternative caspase-independent apoptotic pathways are also delayed in the presence of Bcl-2.³⁹ Post-translational modification of Bcl-2 by MAPKs induces^{40,41} or represses^{42–44} its antiapoptotic activity depending upon cell type, cell cycle stage and apoptosis inducer.

The goal of the present study was to examine the molecular events underlying the transition of pluripotent ES cells to various differentiated lineages. By analyzing the activation profile of MAPKs and the expression pattern of specific genes during the first week of LIF withdrawal, we have characterized an apoptotic pathway triggered upon LIF starvation in ES-derived early differentiated precursor cells. This work also emphasizes novel properties of a p38 inhibitor to study the differentiation processes induced from mouse ES cells.

Results

Activation of MAPKs and caspases upon LIF withdrawal in ES cells

We have previously shown that, upon LIF withdrawal, about 30% of ES cells die by apoptosis during the differentiation process.¹⁶ The loss of DNA integrity in chromatin, reflecting the activation of CAD/DFF (a DNA fragmentation factor) was taken as an index of apoptosis.⁴⁵

The cell death program involves the activation and repression of distinct members of the MAPK family, depending on cell type and apoptotic inducers.²⁹ We have followed the activation profile of MAPK proteins in ES cells grown with or without LIF, using phosphospecific anti-MAPK antibodies that recognize the dually phosphorylated forms of extracellular signal-regulated kinase (ERK)1/ERK2 (P-ERKs), JNK1/JNK2 (P-JNKs) and the different p38 isoforms (P-p38), (Figure 1a). Phosphorylation of one p38 isoform (which

Figure 1 Activation of MAPKs and caspases in ES cells grown in the absence of LIF. (a) Total cell lysates (50 μ g protein; RIPA conditions) from ES cells grown in the continuous presence of LIF (+ LIF) or in the absence of LIF (–LIF) for the indicated time periods in days (d), were analyzed with the phosphospecific anti-p38 (P-p38), anti-ERK1/2 (P-ERKs) and anti-JNK1/2 (P-JNKs) antibodies and with the p38 α (p38), ERK2, JNK1, Bax-1 and PARP-1 antibodies. (b) Total cell lysates as in (a), from adherent (alive) or floating apoptotic (apo) cells grown as indicated were analyzed as in (a) with the p38 antibodies. (c) Genomic DNA was prepared from ES cells grown as monolayers under the indicated conditions in the absence or presence of 40 μ M of the Z-VAD compound. A total of 20 μ g of DNA were loaded on a 2% agarose gel and stained with ethidium bromide after electrophoresis. The reverse photograph of stained DNA is presented, d=day. (d) Total cell lysates as in (a) were analyzed with the anti-Casp3 antibody that recognized both pro-Casp3 (Casp-3) and its active proteolytic cleavage product (cleaved Casp-3)

corresponds to p38 α , see below) correlates with the initiation of the apoptotic process, while phospho-JNKs and phospho-ERKs were not detected during the apoptotic crisis (days 2–5). However, JNK1 and ERK2 phosphorylation was induced 6 days after LIF deprivation at a time where only differentiated cells were growing. We also observed that the activated dually phosphorylated p38 α proteins were mainly present in the apoptotic cell fraction (Figure 1b). These results indicated that p38 α activation was a hallmark of dying cells in LIF-deprived ES cells, while JNK1 and ERK2 are induced after the apoptotic crisis, in living differentiated cells.

We observed no change in the level of the proapoptotic Bax-1 protein. Furthermore, while cleavage of the PARP-1 protein, a target of Casp3, was not detectable upon LIF withdrawal, the broad-range caspase inhibitor Z-VAD-FMK impaired the DNA laddering process, indicating that caspases were activated upon LIF withdrawal (Figure 1c). Surprisingly, and despite the absence of detectable cleaved PARP-1, Casp3 was activated upon LIF withdrawal, in good correlation

with LIF withdrawal-induced apoptosis (Figure 1D). However, PARP-1 cleavage was easily detected in ES cells treated for a few hours with sorbitol, a potent apoptotic inducer,⁴⁶ which triggers Casp3 cleavage and leads to death of 80% of cells. This suggests that our Western blot assay is probably not sensitive enough to detect PARP-1 cleavage in 30% of dead cells (data not shown).

Altogether, these results indicate that a caspase-dependent apoptotic pathway, involving the activation of p38 α MAPK and of Casp3, is induced upon LIF withdrawal in ES cells.

PD169316, but not SB203580, blocks the apoptotic process induced upon LIF withdrawal

The relevance of activated p38 protein in triggering apoptosis was challenged by using two p38 inhibitors, PD169316 and SB203580, known to block the kinase activities of both p38 α and β isoforms.^{32,47–49} Genomic DNA was prepared from ES cells grown in the presence or absence of LIF for 3 days with or without the p38 inhibitors. The onset of apoptosis could be observed after a 3-day LIF withdrawal period, as revealed by the occurrence of typical DNA ladders. At that time, we could clearly observe differentiated adherent and floating apoptotic cells (Figure 2a). By contrast, in the presence of PD169316, cell morphology was less altered and DNA laddering was strongly reduced. Surprisingly, the SB203580 compound does not prevent cell death (Figure 2). The proper functionality of the two p38 inhibitors to block p38 kinase activity was demonstrated in ES cells treated with sorbitol, an apoptotic agent that is a potent p38 inducer.⁵⁰ Indeed, the p38 kinase activity, measured on an exogenous substrate (ATF2), was impaired in the presence of the PD and SB compounds (Materials and Methods) (Figure 2c).

These results indicate that the PD169316 and SB203580 chemicals have differential effects on pathways involved in LIF-withdrawal-induced apoptosis. We have not further investigated the effect of SB203580 on apoptosis, but have undertaken a deeper analysis of the effect of the PD169316 compound on p38 activity, cell survival and differentiation processes.

PD169316 blocks Casp3 cleavage

To reinforce the fact that PD169316 was effective as a p38 inhibitor in our cell system, the phosphorylation state and kinase activity of p38 were monitored in the absence or presence of PD169316. As shown in Figure 3a, p38 phosphorylation, as well as its kinase activity, increased 3 days after LIF withdrawal. In the presence of PD169316, an increase in p38 phosphorylation was observed, but its kinase activity was severely impaired. This observation clearly shows that the PD compound alters the activity of p38 MAPKs. Interestingly and in good agreement with a previous report,⁵¹ we show that p38 phosphorylation was increased in the presence of PD169316, raising the possibility that p38 normally controls its own activity by a negative feedback regulatory loop. The cleavage of Casp3 was also blocked by this compound stressing the point that this inhibitor is a strict antiapoptotic agent.

Figure 2 Differential effects of two p38 inhibitors in ES cells grown in the absence of LIF. (a) Phase-contrast pictures of ES cells cultivated in the presence of LIF (+ LIF), or in the absence of LIF for 3 days (-LIF 3d) with and without 10 μ M PD169316 (PD) or 10 μ M SB203580 (SB), as indicated. Bar: 100 μ m. (b) A total of 20 μ g of DNA from each plate grown under the indicated conditions were loaded on a 2% agarose gel and stained with ethidium bromide after electrophoresis. The reverse photograph of stained DNA is presented. (c) An *in vitro* kinase assay was performed with 300 μ g of total lysates from ES cells grown in the presence of LIF, in the absence (-) or presence (+) of 300 mM sorbitol (S), in the absence (-) or presence (+) of 10 μ M PD169316 (PD) or 10 μ M SB203580 (SB) added to the kinase reaction. The arrow points to the position of the phosphorylated GST-ATF2 (P-ATF₂) exogenous substrate

The effect of this p38 inhibitor on apoptosis was followed by FACScan analysis (Figure 3b). While, as expected, about 30% of the cells were dying by apoptosis, 3 days after LIF withdrawal, in the presence of PD169316, cell apoptosis was reduced to 7%.

P38 α activation is rather a consequence than a cause of apoptosis

The opposing activities of the two p38 inhibitors on apoptosis may be related to the effects of these compounds on other genes or proteins not restricted to the p38 family. To start characterizing the direct effect of p38 isoforms on LIF-withdrawal-induced apoptosis, we used a p38 α ^{-/-} ES cell line in which both alleles of the p38 α gene were deleted by homologous recombination.⁵² This p38 α ^{-/-} cell line behaves as a WT cell line in terms of LIF-induced activation of the STAT3 transcription factor, a pluripotent LIF-dependent cell target¹³

Figure 3 Effect of PD169316 on p38 activity and Casp3 cleavage. **(a)** Total cell lysates (50 μ g) from cells grown under the indicated conditions, in the absence or presence of 10 μ M PD169316 were analyzed with P-p38, p38 and Casp3 antibodies. A measure of 300 μ g of each lysate was used in an *in vitro* kinase assay on the exogenous GST-ATF2 substrate, in the absence or presence of 10 μ M PD169316, further added to the kinase reaction. **(b)** FACSscan analysis of ES cells after PI staining of DNA. DNA contents of ES cells grown under the conditions indicated in **(a)** were plotted as the log of the relative PI fluorescence (proportional to the DNA content) versus the number of cell events. The proportions of apoptotic cells, those in sub-G1 (Apo), are indicated

and contains WT level of the ERK2 kinase (Figure 4a). However, by using the phospho-p38 antibody, which could recognize the four p38 isoforms, we clearly could not observe any p38-activated proteins in the $p38\alpha^{-/-}$, clearly indicating that it is only $p38\alpha$ isoform that is activated upon LIF withdrawal in ES cells (Figure 4b). Thus, to determine if $p38\alpha$ was instrumental in LIF-withdrawal-triggered apoptosis, this cell line, in parallel with a wild-type ES cells, was deprived of LIF for 3 days and apoptosis was followed by the DNA ladder assay (Figure 4c). Apoptosis occurs in $p38\alpha^{-/-}$ upon LIF withdrawal, clearly indicating that the activation of $p38\alpha$, upon apoptosis, is mainly a consequence rather than a cause of apoptosis.

Characterization of genes regulated during differentiation and apoptosis

To identify cell markers whose expression is modulated upon LIF withdrawal, we have undertaken a semiquantitative RT-PCR analysis on candidate genes known to be regulated during apoptosis or cell differentiation (Figures 5 and 6). The number of PCR cycles was optimized for each gene, to keep the amplification reaction linear. The expression profiles of the following genes have been examined over a 7-day period spanning the apoptotic crisis (days 3–5), followed by the expansion of differentiated cells that may secrete LIF and in which the ERK pathway is restored (^{16,53,54}; this study): (i) pluripotent cell markers (repressed expression-1 (*rex-1*), ES

Figure 4 $p38\alpha^{-/-}$ cells die by apoptosis upon LIF withdrawal. **(a)** Western blot analyses were performed with total RIPA cell lysates (50 μ g) from WT or $p38\alpha^{-/-}$ ES cells grown in the continuous presence of LIF (+) or starved for 20 h in medium without LIF (-) and reinduced (+) or not (-) with LIF for 15 min (LIF induction) with the anti-P-Tyr705-STAT3 (P-Tyr-STAT3), anti-STAT3 or anti-ERK2 antibodies as indicated. **(b)** Western blot analyses of total RIPA cell lysates (50 μ g) from WT or $p38\alpha^{-/-}$ ES cells grown in the continuous presence of LIF (+) or without LIF for 3 days (3d) were performed with anti-phospho p38 (P-p38) or anti-p38 α (p38) as indicated. **(c)** Genomic DNA was prepared from a wild-type or $p38\alpha^{-/-}$ ES cells grown as monolayers in the presence (+) or absence of LIF for 3 days (-). DNA was analyzed as indicated in Figure 1

phosphatase (*esp*) and *fgf-4*), (ii) LIF-responsive genes (suppressor of cytokine signaling-3 (*socs-3*), *c-fos* and *junB*), (iii) differentiation-induced cell markers (*cyp-26*, *fgf-5* and Brachyury (*brach*)) and (iv) pro- and antiapoptotic genes from the *Bcl-2/bax* families.^{16,55–59}

Four different patterns of gene expression were observed: (i) genes that are expressed mainly in nondifferentiated ES cells and whose expression is reduced upon LIF withdrawal (*rex-1* and *esp*); (ii) typical LIF-responsive genes, highly expressed in ES cells, whose level of expression decreases quickly upon LIF withdrawal, and that were re-expressed in LIF-secreting differentiated cells (*socs-3*, *c-fos*, *jun-B* and *fgf-4*); (iii) genes whose expression increases upon differentiation and apoptosis (*fgf-5*, *bclx/l* and *bcl-2*); (iv) genes that are

Figure 5 Expression analysis of pluripotency, differentiation and apoptosis gene markers, in ES cells. Total RNA (1 μ g) from ES cells, grown in the presence (+ LIF) or absence of LIF (–LIF) for 1 to 7 days (d), was analyzed by semiquantitative RT-PCR with primers corresponding to the indicated genes. The control lane corresponds to RT-PCR experiments performed without RNA. The RT-PCR reactions (30 or 33 cycles depending on the gene tested) were migrated on a 2% agarose gel and stained with ethidium bromide. Genes have been regrouped (boxes) according to previously defined properties. The reverse photograph of stained DNA is presented

mainly expressed in differentiated cells (*cyp-26* and *brach*). These results revealed that differentiation started as soon as 24 h after LIF withdrawal, with a repression of *rex-1*, *fgf-4* and *esp*. In addition, they showed that *fgf-5* was induced at the beginning of the apoptotic crisis, while *brach* expression was maximal at the end of the crisis. Finally, a novel pattern of expression of some *bcl-2*/*bax* family members was observed, in which the expression of the proapoptotic *bax-1* gene remained constant, in good agreement with the unchanged level of protein (see Figure 1), while *bcl-2* and *bclx/l* expression increases during the apoptotic crisis, within the surviving differentiated cells (see below, Figure 6b).

Identification of genes whose expression is altered by PD169316

We were interested in characterizing PD169316-regulated genes that may be relevant markers involved in differentiation and apoptotic pathways. To this end, we have analyzed the expression profile of genes in cells grown in the absence of LIF and continuous presence of PD169316 for 1–5 days by semiquantitative RT-PCR (Figure 6a). The PD 169316 compound did not impair the proper regulation of *esp*, *rex-1*, *cyp-26* and LIF-responsive genes (*socs-3*, *c-fos*). However, a decrease in the expression of *brach* and *fgf-5* was observed in

Figure 6 Effect of PD169316 on gene expression in ES cells. (a) Total RNA (1 μ g) from ES cells, grown in the presence (+ LIF) or absence of LIF (–LIF) for 1–5 days (d), in the absence or presence of 10 μ M PD169316 (PD), as indicated, was analyzed by semiquantitative RT-PCR, as in Figure 6. The reverse photograph of stained DNA is presented. The PD-regulated genes are bracketed. Groups of genes have been boxed as in Figure 6. (b) Total RNA (1 μ g) from adherent (Alive) or floating apoptotic (Apo) cells, grown in the absence of LIF for 3 days, was analyzed by RT-PCR as in (a). (c) Total RNA (1 μ g) from ES cells grown for 3 days in the absence of LIF, in the absence or presence of PD169316 were analyzed by RTQ-PCR, with primers corresponding to the indicated genes. The FC, corresponding to the level of induction or repression of gene expression in the presence of PD169316, with standard deviations from three independent experiments, are plotted, at scale, for each gene

cells treated with this p38 inhibitor (Figure 6a). In addition, we observed a PD169316-dependent increase of the expression of *bcl-2* (and *bclx/l*, not shown) on the first day of LIF withdrawal, which may explain the cell survival effect of this compound. This possibility was further investigated in experiments where gene expression was monitored on living adherent cells and floating apoptotic cells separately (Figure 6b). *Bcl-2* mRNA was detected in live cells only, while *bax-1* and hypoxanthine phosphoribosyl transferase (*hprt*) mRNA were present in both cell populations, stressing the correlation between *bcl-2* expression and cell survival. In addition, we observed that, in LIF-deprived cells, long-term

treatment with the PD169316 compound (over a 2-week period) led to differentiated cell populations in which apoptosis does not occur (our unpublished results).

Real-time quantitative-polymerase chain reaction (RTQ-PCR) experiments were also carried out, at 3 days after LIF withdrawal in the absence or presence of PD169316 with a subset of primer pairs, including two control (*36B4* and *hprt*), two non-PD-regulated (*bax-1* and *cyp-26*) and two PD-regulated genes (*bcl-2* and *fgf-5*). As shown in Figure 6c, we confirmed, by this approach, that the expression of *bcl-2* (stimulated five-fold) and *fgf-5* (repressed five-fold) was modulated by the PD compound, while the expression of other genes tested was not.

Altogether, these experiments indicate that PD169316-sensitive genes and proteins are regulating particular *fgf-5*- and *brach*-dependent differentiation pathways and that *bcl-2* may preserve the survival potential of the differentiated cell lineages.

Overexpression of Bcl-2 prevents apoptosis induced upon LIF withdrawal

To confirm that the Bcl-2 protein is a PD169316 target involved in the survival of ES-derived differentiated cells, we have established ES cell clones overexpressing an enhanced green fluorescent protein (EGFP)-Flag-Bcl-2 fusion protein (p-fluo-Bcl-2) or the corresponding empty vector. The expression of the fusion protein was directly visualized by fluorescence microscopy and evaluated by Western blot analysis with the anti-Flag and anti-Bcl2- antibodies (Figure 7 and data not shown). Similar numbers of G418-resistant clones were recovered with both constructs. In all, 10 clones derived from each plate were amplified and further characterized. An apoptotic test was performed on five individual G418-resistant clones obtained with each construct. Each clone was plated at low cell density and grown in the presence or absence of LIF for 3 days. The proportion of apoptotic cells was scored after propidium iodide (PI) staining and FACScan analysis (see Materials and Methods). In control pCNX2-transfected clones, the number of cells undergoing apoptosis increased about 10-fold upon LIF withdrawal (see clone 0, Figure 7a). By contrast, in the Bcl-2-overexpressing clones, in which baseline apoptosis was already significantly reduced, the number of apoptotic cells, observed 3 days after LIF withdrawal, remained nonsignificant (clones 1 and 2, Figure 7a). It is worth noting that low levels of Bcl-2 expression (Figure 7b, compare clones 1 and 2) were sufficient to suppress apoptosis. In addition, our observation that an ES cell line overexpressing the EGFP protein alone showed the normal proportion of apoptotic cells upon LIF withdrawal (about 30%) indicated that the prevention of cell death, in the presence of the fusion protein, was directly dependent on Bcl-2 and not on an aberrant effect of the EGFP-fused protein (A Dierich and H Boeuf, unpublished results).

These results, summarized in Figure 8, demonstrate the importance of Bcl-2 in controlling cell survival of early differentiated lineages derived from ES cells upon LIF withdrawal.

Figure 7 Overexpression of Bcl-2 prevents LIF-withdrawal-induced cell death. (a) Phase-contrast micrographs of G418-resistant ES clones expressing the empty vector (clone 0) or the EGFP-Flag-Bcl-2 fusion protein (clones 1 and 2: p-fluo-Bcl-2), grown in the presence or absence of LIF for 3 days, as indicated. Proportions of apoptotic cells (Apo), determined by FACScan analysis after PI staining, are indicated. (b) Western blot analysis, with total RIPA cell lysates (50 μ g) from clones 0, 1 and 2, grown in the presence of LIF, was performed with the anti-Bcl-2 antibody

Discussion

The maintenance of mouse ES cell pluripotency, which is dependent upon the continuous presence of the LIF cytokine, is a complex process that includes pathways involved in cell proliferation, control of genome integrity and inactivation of differentiation programs. Here, we demonstrate that it also involves the repression of a caspase-dependent apoptotic pathway that includes the activation of p38 α MAPK.

During the differentiation process triggered upon LIF withdrawal, about 30% of the cells die by apoptosis, whether growing as monolayers or embryoid bodies (this study and unpublished observations). Apoptosis was demonstrated by three independent criteria: DNA laddering (present study), as well as condensed chromatin observed in cell nuclei by electronic microscopy and positive TUNEL assays (C Hindelang, M Malaisé, D Duval and H Boeuf, unpublished results). The fact that this apoptotic crisis was also observed with clonal ES cells ruled out the possibility that apoptosis was linked to the heterogeneity of the starting cell population (data not shown).

LIF-dependent cell survival may be linked to the antiapoptotic function of STAT3, a LIF-induced transcription factor whose activity is essential for the maintenance of ES cell pluripotency.^{10,60–62} However, the observation that ES cell clones, overexpressing an inducible dominant-negative form

Figure 8 Events occurring during the first week of LIF withdrawal in mouse ES cells, in the presence or absence of PD169316. The modulation of some molecular determinants in response to LIF is depicted: the variation of the phosphorylation state of STAT3 (P-STAT3) and p38 (P-p38), and levels of expression of specific markers are indicated by an 'up' or 'down' arrows. The effect of the PD169316 compound is also indicated

of the STAT3 protein, are not dying by apoptosis prompted us to look for other potential regulators.¹¹

The first week without LIF

The expression of a selected set of genes was followed over a 1-week period after LIF withdrawal during which the apoptotic crisis occurs (between days 3 and 5), followed by the expansion of differentiated cells secreting LIF and in which the ERK pathway is activated^{16,53,54} and this study). The analysis revealed four classes of genes and highlighted novel features of some of them. The *fgf-4* gene, which encodes a factor involved in different processes, like the propagation of cells from the ICM and trophoblasts of the embryo and limb patterning,^{9,63,64} was reinduced in differentiated cells and behaved as an LIF-responsive gene. Also, the kinetics of expression of *fgf-5* (a specific marker of primitive ectoderm and of some of its derivative),^{65–67} and of *brach* (a mesoderm marker) mimicked the *in vivo* situation in early mouse embryos: the decrease of *fgf-5* expression precedes the establishment of mesoderm that correlates with an increased expression of the *brach* gene.^{68–70}

The downregulation of the expression of various markers within the first 24 h of LIF withdrawal, which was not affected by the presence of the p38 inhibitor (PD169316), indicates that cells are committed to differentiate as soon as LIF was withdrawn, even in the presence of the inhibitor. This also indicates that apoptosis, observed 3 days after LIF withdrawal, occurs in differentiated cell lineages. From this study, it is not yet clear if particular cell lineages are dying by apoptosis or if a general stress-like process is triggered upon LIF withdrawal. Further characterization of specific cell lineage markers differentially expressed in apoptotic and in living differentiated cells will help answer this question. Nevertheless, we report on a novel function of Bcl2 that plays a key role in protecting early differentiated precursors against cell death.

p38 on the stage: new properties of p38 inhibitors

During apoptosis, differential activation of MAPKs has been reported in many instances: the proapoptotic functions of JNKs and p38 MAPKs, and antiapoptotic role of ERKs were first reported on PC12 cells deprived of NGF;²⁷ by contrast, protective antiapoptotic effects of JNKs and p38 in cancer or neuronal cells^{71,72} as well as activation of ERKs upon cell detachment-induced apoptosis, have also been described.⁷³ This indicates that apoptosis-dependent induction of MAPKs is both inducer and cell-type specific. Caspase activation, upon stress-induced cytochrome c release, leads to the cleavage of particular substrates such as the PARP-1 protein.^{74,75} The p38 MAPK can also directly activate cytochrome c release and induce apoptosis, independently of caspase activation.⁷⁶

We described here an apoptotic pathway that correlates with the activation of p38 α MAPKs and of Casp3. However, by using a p38 α ^{-/-} cell line, we demonstrate that the activation of p38 α is not critical for the triggering apoptosis, but is a secondary event. This pathway may be related to the p38 α -independent stress-induced apoptosis.⁵²

We have identified novel properties of the p38 inhibitor PD169316, which blocks the cleavage of the effector Casp3 and induces cell survival without impairing cell differentiation. This is the first report on cell growth conditions allowing to dissociate early cell differentiation and apoptosis. Furthermore, the repression of *fgf-5* and *brach* expression, in the presence of this inhibitor, suggests that PD169316-treated cells might be altered in their differentiation potential. In contrast to PD169316, the SB203580 compound, widely used to study p38 function, does not inhibit the apoptotic process. In the presence of SB203580, ES cells behave as p38 α ^{-/-} cells stressing that SB203580 is a *bona fide* p38 α inhibitor. However, the fact that the proportion of apoptosis was slightly higher in the presence of SB than in the p38 α ^{-/-} cell line indicates that this compound may stimulate directly proapoptotic proteins, not yet identified. Despite the fact that our work, in agreement with others,^{34,35,77} questions the selectivity of these compounds towards p38 kinases, it depicts new (anti- and proapoptotic) cell growth conditions that will be helpful to characterize genes involved in early cell differentiation and apoptosis. As an example, we showed that the forced expression of *bcl-2* in ES cells, which mimics the PD-induced

bcl-2 expression, protects the differentiated ES cell derivatives against apoptosis.

In conclusion, while the LIF-dependent activation of STAT3, and/or high level of OCT4 and NANOG proteins, keeps ES cells alive and pluripotent, the fine tuning of the expression of members of the Bcl-2 family becomes critical for the survival of early differentiated precursor cells, upon LIF withdrawal (Figure 8). The further characterization of PD169316- and SB203580-sensitive kinases and corresponding substrates will help identify the connections between cell differentiation and apoptosis, two crucial events that occur within the ICM of blastocysts during normal embryogenesis.

Materials and Methods

Cells and reagents

The S1 ES cell line, derived from mouse 129 strain,⁷⁸ grown in the absence of feeder cells in a LIF-supplemented STEM medium (STEM medium: high glucose DMEM (Invitrogen Corporation); 10% FCS (Euromedex); 0.1 mM β -2-mercaptoethanol (Sigma), supplemented with 500 pM LIF) were passaged or the medium was changed every other day. The *p38 α* ^{-/-} cell line, in which both alleles of the *p38 α* gene have been targeted by homologous recombination, has been described previously.⁵²

The anti-ERK2 (C-14), anti-Bax-1 (Δ 21), anti-p38 (N20), anti-STAT3 (F2) (Santa Cruz Biotechnology), anti-JNK1, anti-Bcl-2 (Pharmingen), anti-phospho JNKs (Promega), anti-phospho p38, anti-Casp 3, (Cell signaling Technology), anti-phospho ERKs (Biolabs) and anti-phospho-Tyr 705 STAT3 (Biosource International) antibodies were used as recommended by the manufacturers. The PARP-1 antibody was a generous gift from J de Murcia (ESBS, Strasbourg). The PD169316 and SB203580 were from Calbiochem, Z-VAD-FMK was from Biomol Research Laboratory and the p38 MAPK assay kit was from Cell Signaling Technology.

Apoptotic test

ES cells were plated at low cell density (10^5 cells/ml) in a LIF-containing STEM medium. The day after, cells were washed with PBS and fed with STEM supplemented or not with LIF for 3 days. Apoptotic cells were detected by light microscopy and their proportion was determined after PI staining, by FACScan analysis (see below). For DNA ladder detection, genomic DNA was prepared as described.¹⁶

Kinase and caspase inhibitors or inducer

PD169316, SB203580 or Z-VAD treatments: At 24 h after plating of ES cells in an LIF-containing STEM medium, the plates were rinsed in STEM medium and maintained in LIF-containing or LIF-free STEM medium, with or without the different chemicals (10 μ M PD169316, 10 μ M SB203580 or 40 μ M Z-VAD, prepared in DMSO), for the indicated time periods, at which cell lysates and genomic DNA were prepared.

Sorbitol treatment: At 24 h after plating of ES cells in LIF-containing STEM medium, cells were treated in the presence of LIF with 300 mM sorbitol. Cell lysates were prepared 3 h after treatment.

Cell lysates and Western blots

Cytosolic and nuclear cell lysates were prepared as described.⁷⁹ Whole-cell lysates were a mixture of cytosolic and nuclear lysates at a 2 : 1 ratio. Cells were also lysed in RIPA buffer (PBS, 0.1% SDS, 0.5% Na

deoxycholate, 1 μ g/ml protease inhibitor cocktail, 1 mM Pefabloc, 20 mM NaF, 1 mM Na vanadate). Viscous lysates were passed several times through a 22-gauge needle and centrifuged 10 min at 10 000 r.p.m. The total cell lysates were resolved by SDS-PAGE (10 or 12% (for Casp3)) and electrotransferred onto nitrocellulose membranes in the presence or absence (for Casp3) of 0.07% SDS. Proteins were reacted with the different antibodies, as recommended by the manufacturers.

In vitro kinase assay

300 μ g of total cell lysates (mix of 1/3 nuclear and 2/3 cytosolic fractions⁷⁹) were immunoprecipitated with 15 μ l of the agarose-immobilized anti-phospho-p38 MAPK (Thr 180/Tyr 182) antibody (p38 MAPK assay kit, Cell Signaling Technology) overnight at 4°C. The recovered beads were washed twice with 500 μ l of lysis and kinase buffers and then processed as described in the kit protocol. The kinase reactions were performed in the presence or absence of 10 μ M PD169316 or 10 μ M SB203580.

Flow cytometry analysis of cell cycle

ES cells were washed once with PBS, trypsinized and collected by centrifugation. After overnight fixation with 75% ethanol at 4°C, pelleted ES cells were resuspended in PBS and incubated with 100 μ g/ml of RNase A at 37°C for 30 min. Cells were stained with 15 μ g/ml PI for 30 min at room temperature and analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Data acquisition and analysis were performed using CellQuest software. The proportion of cells in sub-G1 corresponds to apoptotic cells. Counting was made on 30 000 cells for each sample.

Semiquantitative RT-PCR and RTQ-PCR

The total RNA from adherent ES cells was prepared with the Qiagen column kit (Qiagen) and treated with DNase (5 U/100 μ g RNA, Sigma). When indicated, the floating apoptotic cells were recovered by centrifugation prior to cell lysis and RNA preparation. The total RNA (5 μ g) was reverse-transcribed (RT) with random hexameric primers and the MLV Reverse Transcriptase (Sigma). The RT reaction products were used for PCR reactions with specific primer sets as described previously.¹⁶ PCR reactions were performed with primers corresponding to the LIF-independent *hprt* gene with each RNA preparation. PCR products were analyzed on 2% agarose-gel after ethidium bromide staining. The number of PCR cycles was optimized for each gene, to keep the amplification reaction linear.

For RTQ-PCR experiments, performed with the Light Cycler system (Roche, Idaho Technologies), 3 μ l of the RT reaction (the 1/7th of the original reaction) was mixed in the PCR buffer containing 4 mM MgCl₂, 0.25 mM dNTP, 0.25 mg/ml BSA, 0.5 μ l of the SYBR Green I dye (diluted 1000 $\times$ from the stock), 0.5 μ l of Taq Pol (Sigma) and 2.5 μ M final of each primer. Amplifications were performed as follows: a 20 s denaturation at 95°C, 40 cycles (1 s at 95°C, 20 s at 59°C, 15 s at 72°C, 1 s for fluorescence lecture (performed between 82 and 88°C, depending upon the primer pairs)). After amplification, to determine the specificity of the PCR products, a melting curve was acquired by heating the product at 95°C, cooling at 70°C for 20 s and then remelting slowly (0.3°C/s) up to 95°C. In addition, the correct size and purity of each PCR product were verified. The Cp value (minimum cell cycle number in the linear zone of amplification) was determined for each gene in these two conditions: 3 days without LIF (J3) and 3 days without LIF + 10 μ M PD 169316 (J3PD). The fold change (FC), corresponding to the level of induction or repression

of gene expression in the presence of PD169316 is calculated with the formula: $2^{[C_p(J3)-C_p(J3PD)]}$.

The primers used for the *esp*, *socs-3*, *c-fos*, *junB* and *36B4*,¹⁶ *rex-1*, *brach*, *hprt*,⁸⁰ and *cyp-26*⁸¹ have been described. GenBank accession numbers and primers used for the other genes of interest are as follows: *fgf-4* (M30642): 5'-GGGGCACGGCTGGGACGGCTTGGTGGCCCGC, 5'-CCCCTTCTTGGTCCGCCGTTCTTACTGAGGGCC; *fgf-5* (M30643): 5'-GCGGCTCGGAACATAGCAGTTTCCAGTGGAGCCC, 5'-GCCTCTCTTGGCTTTCCCTCTCTTGTTCAGGGCC; *bax-1* (L22472): 5'-GGGGCCTTTTGGCTACAGGGTTTCATCC, 5'-GCCACAAAGATGGTCACTGTCTGCC; *bclx/l* (L35049): 5'-GGGTGTACCTGCTTGTGGTGCCTGG, 5'-CCCGCGCATCCAACTGCTGCTGTGGC; and *bcl-2* (M16506): 5'-GGCTGCCAGGACGTCTCCTCTCAGGCCCC, 5'-CCG TTATCCTG-GATCCAGGTGTGCAGATGCC.

All experiments reported in the present study have been repeated at least three times.

Construction of expression vectors

p513-kEGFP-HA vector (a gift from Dr. M Vigneron, ESBS, Illkirch): the 736-bp fragment, containing the EGFP c-DNA, was PCR amplified from the pCXN-EGFP vector⁸² with a 5' primer containing an *AvrII* restriction site and a 3' primer containing an *NheI* restriction site, and cloned into the *NheI* site of an SV40-based vector, pHA (a gift from Dr. B Chatton, ESBS, Illkirch;⁸³ this vector is similar to p513-HA¹⁶). A Kozak sequence (K), 5'-GCCGCC-3' was added by directed mutagenesis at the proper location upstream of the ATG of the EGFP sequence.

p513-Flag-Bcl-2 vector: Bcl-2 α c-DNA, obtained after RT-PCR amplification (of mRNA from ES cells that had been treated for 3 days in the presence of PD169316), with the 5' primer including an *NdeI* restriction site and the 3' primer including an *HindIII* restriction site, was subcloned between the *NdeI*/*HindIII* sites of the p513-Flag vector, which was derived from the p513-HA vector by replacing the HA tag by the Flag tag.

p513-kEGFP-Flag-Bcl-2 vector: the *EcoRI*-*NheI* kEGFP fragment from p513-kEGFP-HA vector was subcloned between the *EcoRI*-*NheI* sites of the p513-Flag-Bcl-2 vector.

pCXN2-EGFP-Flag-Bcl-2 (p-fluo-Bcl-2) vector encodes the EGFP-Flag-Bcl-2 fusion protein: the blunted *EcoRI*-*HindIII* fragment of the p513-kEGFP-Flag-Bcl-2 (including kEGFP-Flag-Bcl-2 fragment) was subcloned into blunted *XhoI* site of the pCXN2 vector that includes a TK-neo selection cassette (a vector previously called pCAGGS-neo⁸⁴).

Stable ES clones expressing EGFP fusion proteins

To establish ES cell clones expressing the EGFP-Flag-Bcl-2 fusion protein, ES cells, maintained in an LIF-containing STEM medium on 9 cm Petri dishes, were transfected with 6 μ g of the p-fluo-Bcl-2 or pCXN2 vectors with Exgen 500 (Euromedex). After 2 weeks of selection in the presence of 200 μ g/ml of G418, EGFP/G418- and G418-positive clones were recovered and grown in the constant presence of G418. Pictures of clones (Leica microscope) were recorded by computer acquisition using the Coolsnap software.

Acknowledgements

We thank A Dierich and the staff of the ES cell facility for their expert advice and materials, J and G de Murcia for the anti-PARP-1 antibody, Dr. Gabel, at Pfizer Inc., who provided us with the p38 α ^{-/-} cell line,

D Metzger and M Okabe for the pCXN-EGFP vector, Dr. Miyazaki for the pCXN2 expression vector and B Chatton and M Vigneron for the gift of materials, C Hindelang for her expertise in some of the unpublished experiments, J Barths for the help with the FACScan analysis, D Hentsch for good advice concerning the use of the coolsnap software and N Ghyselinck for fruitful discussions. We are grateful to the staff of the cell culture, biocomputing and artwork facilities for providing help and material at IGBMC. HB wishes to thank JF Moreau, JL Taupin, J Dechanet, V Pitard and S Daburon, at the UMR 5164-CNRS-CIRID, Bordeaux, for their support concerning the revised version of this article. This work was funded by the Centre National de la Recherche Scientifique (CNRS), the Institut National de la Santé et de la Recherche Médicale (INSERM), the Centre Hospitalier Universitaire de Strasbourg, the Association pour la Recherche sur le Cancer (ARC), the Ligue Nationale contre le Cancer (comité national et comité de la Gironde) and the Université Louis Pasteur de Strasbourg. DD was a recipient of a fellowship from CNRS and ARC.

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