

Regulation of the human leukaemia inhibitory factor (LIF) promoter in HEC-1B endometrial adenocarcinoma cells

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Leukaemia inhibitory factor (LIF) is a pleiotropic cytokine which has been found to be expressed in the human endometrium and to play an important role in human reproduction. In the present study we investigated expression and regulation of the human LIF promoter in HEC-1B endometrial adenocarcinoma cells using a luciferase reporter plasmid bearing a 666 bp promoter fragment (h666LIF-Luc) in transient transfection assays. HEC-1B cells were first shown by reverse transcription–polymerase chain reaction (RT–PCR) to be able to produce endogenous LIF mRNA. The LIF promoter was efficiently transcribed in HEC-1B cells, showing much higher levels of basal activity than in the previously studied Jurkat T-lymphoma cells and SKUT-1B uterine mesodermal tumour cells. The activity of the LIF promoter was stimulated in HEC-1B cells by a combination of phorbol ester (TPA) and ionomycin, which we had previously found to strongly induce its activity in Jurkat T-lymphoma cells. We next studied the effect of progestin (medroxyprogesterone acetate; MPA) on the LIF promoter activity in HEC-1B cells. The LIF promoter was not stimulated by MPA treatment in the presence of transfected progesterone receptor B (PR-B) expression vector in HEC-1B cells, while we had previously described its induction by MPA in SKUT-1B cells. This indicates that progestin-dependent regulation of the LIF promoter in uterine tumour cells is different in cells of epithelial and mesodermal origin.

Key words: endometrium/leukaemia inhibitory factor/plasmid transfection promoter/tumour cells

Introduction

Leukaemia inhibitory factor (LIF) was first described based on its capacity to induce differentiation and death of M1 murine leukaemia cells (Metcalf, 1992). Subsequently, LIF was shown to be a polyfunctional cytokine (for a review see Gearing, 1993). Recent studies indicate that LIF plays an important role in reproduction. It has been shown to be expressed in the murine preimplantational blastocyst (Conquet and Brulet, 1990) and to be produced in the rodent uterus under maternal control with maximum activity at around the time of implantation (Bhatt *et al.*, 1991). Implantation does not occur in LIF knock-out mice (Stewart *et al.*, 1992), indicating that LIF is essential for this process. LIF has also been found to be expressed in the human endometrium, with the highest levels during the progesterone-dominated secretory phase (Charnock-Jones *et al.*, 1994; Kojima *et al.*, 1994; Arici *et al.*, 1995; Chen *et al.*, 1995; Vogliagis *et al.*, 1996). We have previously described progestin-dependent stimulation of LIF-promoter activity in SKUT-1B uterine tumour cells of mixed mesodermal origin (Bamberger *et al.*, 1997b). On the other hand, in Jurkat T-lymphoma cells, progestin treatment had no effect on LIF promoter activity (Bamberger *et al.*, 1997b). In these cells, the LIF promoter was strongly induced by a combination of phorbol ester (TPA) and calcium ionophore (ionomycin) (Bamberger *et al.*, 1997a).

In the present study, we analysed the activity of the human LIF promoter in HEC-1B uterine tumour cells of epithelial

origin (endometrial adenocarcinoma cells), using a luciferase reporter construct bearing 666 bp of the human LIF promoter (h666LIF-Luc) in transient transfection assays.

Materials and methods

Cell culture

HEC-1B, SKUT-1B and Jurkat cells were purchased from ATCC (Rockville, MD, USA). HEC-1B cells were cultivated in Dulbecco's minimal essential medium (DMEM), SKUT-1B cells were cultivated in a 1:1 mix of Ham's F12 and DMEM and Jurkat cells were cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium (all from Gibco BRL, Gaithersburg, MD, USA), with 10% fetal calf serum (FCS) and antibiotics, and passaged twice weekly. Normal peripheral blood lymphocytes (PBL) were isolated from heparinized venous blood from healthy volunteers (after obtaining informed consent), using Ficoll–Isopaque density centrifugation, and were kept for 24 h in culture in RPMI 1640 medium with 10% FCS and antibiotics before stimulation and RNA extraction.

RNA extraction

Total RNA from stimulated (TPA 10^{-7} M + ionomycin 1 µg/ml for 24 h) and unstimulated HEC-1B and PBLs was extracted using the RNazol method (Tel-Test, Friendswood, TX, USA). RNA was extracted with chloroform (0.2 ml/2 ml homogenate), precipitated with isopropanol, washed with 75% ethanol, and dissolved in diethylpyrocarbonate (DEPC)-treated, RNase-free water. RNA concentration and purity were determined by spectrophotometry.

RT-PCR and DNA sequencing

Complementary DNA was synthesized from 5 µg total RNA with SuperScript RNase H⁻ reverse transcriptase (RT) (Gibco BRL) using oligo(dT) primers (Pharmacia, Piscataway, NJ, USA). Of the resulting cDNA, 5% was used as a template for polymerase chain reaction (PCR). PCR was carried out in the presence of 5 pmol/µl 5'- and 3'-primers, 10 mM dNTPs, 2.5 IU of *Pfu* DNA polymerase (Stratagene, Heidelberg, Germany) in a final volume of 50 µl. The PCR conditions were as follows: 30 cycles (melting, annealing, and extension temperature: 94, 60 and 72°C respectively) were performed using the following LIF-specific primers: primer 1 (5' primer): 5'-GGCCCCG-ACACCCATAGACG-3', primer 2 (3' primer): 5'-CCACGCGC-CATCCAGTAAA-3', amplifying a 455 bp fragment. Amplifications with human glyceraldehyde phosphate dehydrogenase (GAPDH)-specific primers served as internal controls. PCR products were electrophoresed in a 1% agarose gel.

The PCR product from HEC-1B cells was isolated from a 2% low-melting agarose gel and blunt-end-ligated into pCR-Script SK (+) (Stratagene) and sequenced by the dideoxy chain termination method using [³⁵S]-dATP (Amersham, Arlington Heights, IL, USA). Sequencing reactions were analysed on 6% polyacrylamide wedge gels and visualized by autoradiography.

Cloning of the human LIF promoter into pGL3-basic

Amplification of the 666 bp human LIF promoter fragment from genomic DNA and cloning into pGL3-basic has been described elsewhere (Bamberger *et al.*, 1997a).

Transfection experiments and luciferase assay

HEC-1B and SKUT-1B cells were plated in 12-well plates (Costar, Cambridge, MA, USA) at a density of 2 × 10⁵ cells/well. After 24 h, cells were transfected by the lipofection method using N-[1-(2,3-dioleoyl)propyl]-N,N,N-trimethylammonium methyl sulphate (DOTAP reagent; Boehringer Mannheim, Mannheim, Germany). Jurkat cells were transfected by electroporation and plated in 12-well plates at a density of 500 000 cells/well. The transfection mixture contained 1 µg reporter plasmid/well and, when necessary, 0.4 µg expression vector (PR-B or corresponding mock vector)/well. 8 h after transfection, cells were refed with Phenol Red-free medium containing 10% charcoal-stripped FCS to avoid steroid contamination. After 12 h, the cells were stimulated with TPA (10⁻⁷ M, Sigma, Deisenhofen, Germany) and ionomycin (1 µg/ml, Sigma) for 8–12 h or with medroxyprogesterone acetate (MPA, 2.5 × 10⁻⁷ M; Sigma, Deisenhofen, Germany) for 36 h. At 44 h after transfection, the cells were washed, pelleted, and lysed with reporter lysis buffer (Promega, Madison, WI, USA). After one freeze–thaw cycle, luciferase activity in the lysate was determined in a luminometer (Lumat LB 9501; Berthold, Wildbad, Germany). All experiments were repeated at least three times in triplicates.

Results

HEC-1B endometrial adenocarcinoma cells express LIF mRNA

Expression of endogenous LIF mRNA in HEC-1B cells was evaluated by RT-PCR. Figure 1 (upper panel) shows the results of an RT-PCR amplification with human LIF-specific primers. A band of the expected length (455 bp) is obtained with cDNA from stimulated normal peripheral blood lymphocytes (PBL) used as a positive control. A band of the same length was obtained from HEC-1B endometrial adenocarcinoma cells.

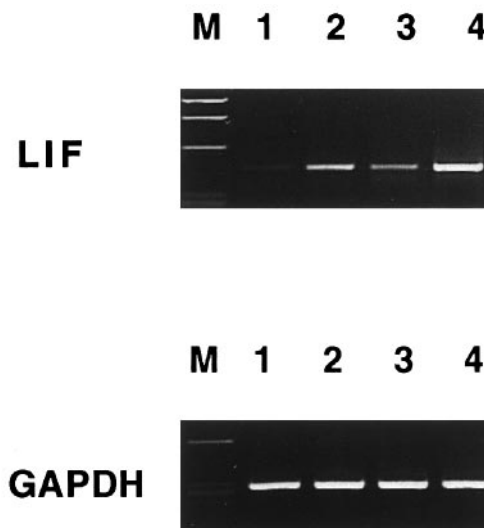


Figure 1. Expression of leukaemia inhibitory factor (LIF) mRNA in HEC-1B cells. Ethidium bromide gel showing the results of reverse transcription–polymerase chain reaction (RT–PCR) amplification with human LIF-specific primers (upper panel) and glyceraldehyde phosphate dehydrogenase (GAPDH)-specific primers (lower panel), used as an internal control. Lanes M = molecular weight marker; lane 1 = unstimulated HEC-1B cells; lane 2 = HEC-1B cells stimulated with phorbol ester (TPA) and ionomycin; lane 3 = unstimulated peripheral blood lymphocytes (PBL); lane 4 = PBL stimulated with TPA and ionomycin (positive control).

The HEC-1B PCR product was subcloned into pCR-Script and DNA sequencing confirmed its identity with the published LIF sequence. Thus, HEC-1B cells are able to express a low amount of LIF mRNA and are a good model for studying LIF regulation. In addition, the intensity of the band in HEC-1B cells stimulated with TPA and ionomycin was increased, showing a similar response as in stimulated cells of lymphocytic origin. While this is not a quantitative result, it appears to represent a tendency, which was confirmed by the transfection experiments. Amplification with GAPDH-specific primers (Figure 1, lower panel) served as an internal control.

Basal activity of the h666LIF promoter in HEC-1B cells

Transient transfection experiments were performed with the h666LIF-Luc construct in HEC-1B endometrial adenocarcinoma, SKUT-1B mesodermal uterine tumour and in Jurkat T-lymphoma cells. The results are presented in Figure 2. The levels of activity were compared with the levels of activity of the 'empty' pGL3-Luc vector, into which the LIF promoter has been cloned. Constitutive expression from the LIF promoter in HEC-1B cells was 14.4 times greater than expression of the pGL3-Luc basic vector, and was thus higher than in SKUT-1B cells (3.3 times greater than pGL3-Luc) and Jurkat cells (2.3 times greater than pGL3-Luc), where the promoter was previously shown to have low basal activity (Bamberger *et al.*, 1997a).

Stimulation of h666LIF-Luc expression by TPA and ionomycin

Since stimulation by TPA and ionomycin strongly induced expression of the LIF promoter in Jurkat T-lymphoma cells,

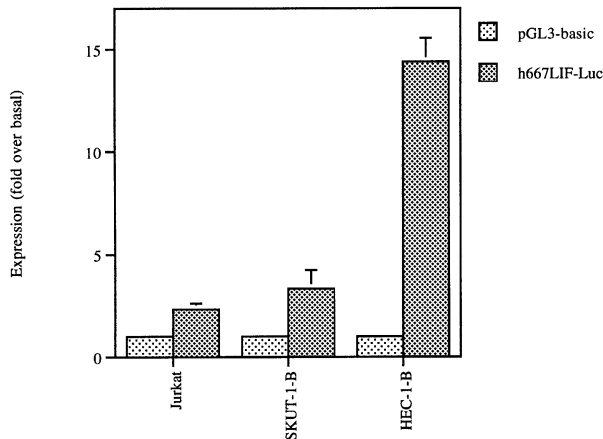


Figure 2. Basal activity of the h666LIF promoter in HEC-1B, SKUT-1B and Jurkat cells. h666LIFG-Luc was transiently transfected into Jurkat, SKUT-1B and HEC-1B cells and the levels of basal activity of this construct 48 h after transfection are presented in comparison with the levels of basal activity of the pGL3-Luc vector, into which the leukaemia inhibitory factor (LIF) promoter has been cloned. Experiments were performed in triplicate and repeated three times. Results are expressed as mean \pm SD.

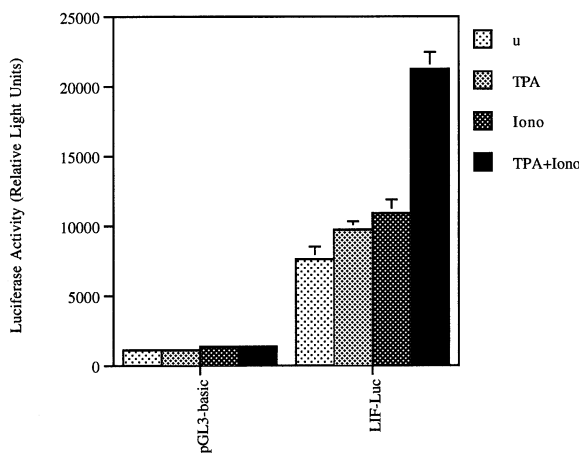


Figure 3. Stimulation of leukaemia inhibitory factor (LIF) promoter activity by phorbol ester (TPA) and ionomycin. h666LIF-Luc was transiently transfected into HEC-1B cells. For the next 8 h, the cells were either left unstimulated (u), or stimulated with TPA, ionomycin (iono), or both (TPA + Iono). No induction was observed when TPA or ionomycin were used alone. Combined treatment was followed by stimulation of LIF promoter activity. No induction of activity was observed with the control vector (promoter- and enhancer-less pGL3-Luc). Experiments were performed in triplicate and repeated three times. Results are expressed as mean \pm SD.

we next investigated the effect of these factors on LIF promoter activity in HEC-1B cells. The results are presented in Figure 3. Following 8 h of treatment with TPA or ionomycin, no induction of LIF promoter activity could be observed. Only combined treatment with both substances induced stimulation of LIF promoter activity. This induction was less pronounced than the one we had found in Jurkat T-lymphoma cells (Bamberger *et al.*, 1997a). On the other hand, as shown in Figure 2, the level of basal activity of the LIF promoter in HEC-1B cells was much higher than in Jurkat cells, indicating that these cells contain activating factors for the LIF promoter in the unstimulated state.

Effect of progestin treatment on LIF promoter activity

Expression of LIF in uterine tissues seems to be under maternal control and is enhanced during the progesterone-dominated second half of the cycle. We previously found that, in SKUT-1B uterine tumour cells of mixed mesodermal origin, the activity of the LIF promoter can be induced by MPA treatment in the presence of a co-transfected PR-B expression vector (Bamberger *et al.*, 1997b). We were now interested to see if this was also the case with uterine tumour cells of epithelial origin, such as the HEC-1B endometrial adenocarcinoma cells. Since these cells, like the SKUT-1B cells, are devoid of functional PR, we co-transfected the h666LIF-Luc vector with either an expression vector for the PR-B, or an appropriate empty vector (mock). Treatment with MPA (2.5×10^{-7} M) for 36 h did not result in induction of the LIF promoter in HEC-1B cells (Figure 4A), as was the case with SKUT-1B cells (Figure 4B). To exclude that the absence of an effect on the LIF promoter in HEC-1B cells might be due to a defect in PR-B expression or function in HEC-1B cells, the effect of the transfected PR-B was tested in parallel experiments on a PRE-Luc reporter plasmid, of which a 10-fold induction in the presence of MPA was observed, as expected (data not shown). This indicates that the transfected PR-B is functional in HEC-1B cells.

Discussion

In the present study, we investigated the activity and regulation of the human LIF promoter in HEC-1B uterine tumour cells of epithelial origin (endometrial adenocarcinoma cells). First, expression of endogenous LIF mRNA in HEC-1B cells was investigated by RT-PCR using LIF-specific primers. A band of the appropriate length was obtained (Figure 1), and sequencing after subcloning into pCR-Script confirmed identity of the product with the published LIF sequence. Thus, the HEC-1B cells produce a low amount of endogenous LIF mRNA and are a good model to study the regulation of the LIF promoter in uterine cells of epithelial origin.

We next performed transient transfection experiments using a 666 bp fragment of the human LIF promoter inserted into the pGL3-luciferase reporter plasmid (h666LIF-Luc). This construct was efficiently transcribed in HEC-1B cells, where its levels of basal activity were higher than those observed with two other previously studied cell lines, the Jurkat T-lymphoma and the SKUT-1B uterine mixed mesodermal tumour cells (Figure 2). We then assessed whether the LIF promoter activity in HEC-1B cells can be induced by phorbol ester (TPA) alone, or in combination with the calcium ionophore ionomycin, which had successfully induced its activity in Jurkat T-lymphoma cells (Bamberger *et al.*, 1997a). We detected stimulation of the LIF promoter when these substances were used in combination (Figure 3), but no effect when either of them were used alone. This induction, while significant, was not as strong as the one seen in Jurkat cells (Bamberger *et al.*, 1997a). On the other hand, as shown in Figure 2, the level of basal activity of the LIF promoter is much higher in HEC-1B cells when compared with Jurkat cells. This indicates that in HEC-1B cells activating transcription factors are able

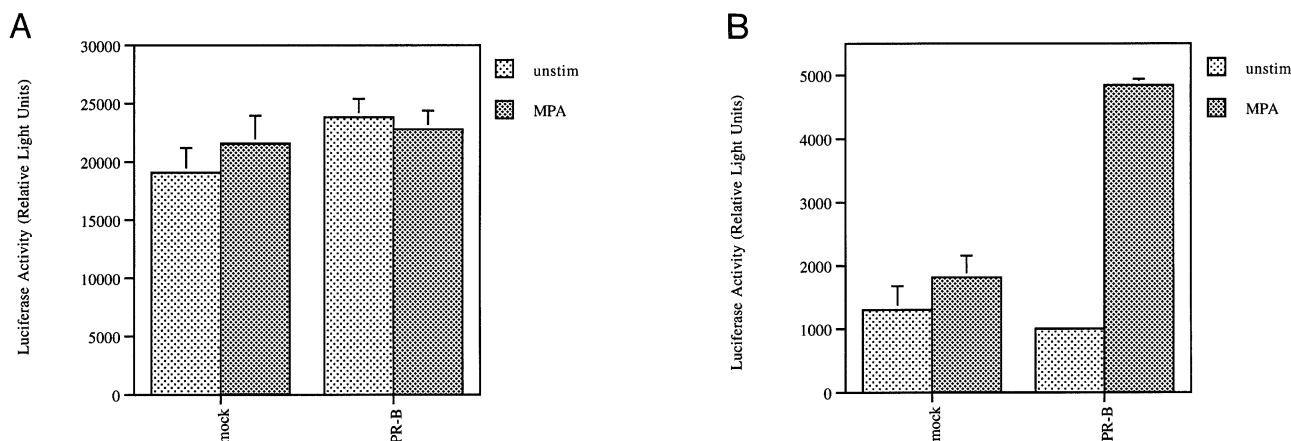


Figure 4. Effects of medroxyprogesterone acetate (MPA) on expression of the leukaemia inhibitory factor (LIF) promoter in HEC-1B cells. (A) HEC-1B or (B) SKUT-1B cells were co-transfected with h666LIF-Luc and an expression vector for PR-B or a corresponding empty vector (mock). The cells were either left unstimulated, or stimulated with MPA (2.5×10^{-7} M) for 36 h. Experiments were performed in triplicate and repeated three times. Results are expressed as mean \pm SD.

to induce LIF promoter activity in the absence of stimulation. This is not the case with Jurkat cells, where the activity of the LIF promoter might be inhibited in unstimulated cells, and only activation of the cells by TPA and ionomycin results in levels of activity which are comparable with those obtained in unstimulated HEC-1B cells (Bamberger *et al.*, 1997a). TPA and ionomycin act by activating protein kinase C (PKC) (Blumberg *et al.*, 1984; Nishizuka, 1984). It is interesting to observe at this point that a recent study of the PKC isoform expression and activation pattern in HEC-1B cells (Bamberger *et al.*, 1996) indicated that these cells have very high levels of expression of all investigated PKC isoforms, and that some of these (i.e. PKC α , β , and δ) were found to be constitutively active in unstimulated cells, which might implicate them in inducing the high levels of basal activity of the LIF promoter observed in this study. A computer analysis performed with the 666 bp LIF promoter (TFSEARCH ver.1.3) indicated that, while there are no classical AP-1 response elements to account for the induction by TPA and ionomycin, several putative c-ets and NF- κ B binding sites are present in this region, which might be implicated in this type of activation (for more details see Bamberger *et al.*, 1997a).

We were next interested to determine whether the LIF promoter can be stimulated by progestin treatment in HEC-1B cells, as had been the case for the SKUT-1B uterine tumour cells, but not for the Jurkat T-lymphoma cells (Bamberger *et al.*, 1997b). We found no effect of MPA treatment on LIF promoter activity in HEC-1B cells, although an expression vector for the PR-B had been co-transfected and was functioning efficiently, as indicated by MPA induction of a PRE-Luc reporter plasmid (Figure 4A,B). A previous study (Arici *et al.*, 1995) also did not find direct effects of oestradiol, progesterone or MPA on LIF mRNA expression by cultivated endometrial cells and postulated that steroid hormones might be acting by way of paracrine/autocrine factors to modulate LIF expression. On the other hand, steroid receptor status of these cells is not described. Also, when placing the data we obtained in HEC-1B cells into a patho-physiological context, one should keep in mind that, although epithelial in origin,

they are tumour cells. Interestingly, Arici *et al.* (1995) also observed higher constitutive and relatively less regulated levels of LIF mRNA in endometrial gland cells when compared with stromal cells, which correlates very well with our data on constitutive expression in HEC-1B cells when compared with SKUT-1B cells of mesodermal origin.

The clinical importance of LIF expression in the human endometrium is indicated by the fact that endometrial explant cultures from infertile women presenting repeated failures of embryonic implantation or unexplained primary infertility were significantly lower when compared with fertile women (Delage *et al.*, 1995). In addition, it has recently been indicated that LIF might play an important role in the pathogenesis of certain kinds of solid tumours, including steroid-dependent breast cancers (Estrov *et al.*, 1995; Kellokumpu-Lehtinen *et al.*, 1996). For these reasons, understanding the molecular basis of LIF promoter regulation in tumour cells of uterine origin may have therapeutic implications.

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