Role of leukemia inhibitor factor (LIF) in decidualisation of murine uterine stromal cells *in vitro*

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Abstract

Decidualisation of uterine stromal cells is a prerequisite for implantation of the embryo in mice. Here we have used an *in vitro* culture system in which stromal cells decidualise as indicated by a number of markers, including an increase in alkaline phosphatase (ALP) activity. The latter was used as a quantitative marker of decidualisation in the presence of low (2%) fetal calf serum. Prostaglandin E₂ (PGE₂), which is known to induce decidualisation, increased ALP activity, and this effect was blocked in a dose-dependent manner by indomethacin. Leukemia inhibitory factor (LIF) was then examined, but it had no effect on PGE₂ secretion. However, LIF suppressed ALP activity in a dose-dependent manner in the presence of 2% serum, while an inhibitor of LIF that competes for binding to its receptor reversed the effect of LIF and increased ALP activity above the control level. In serum-free cultures,

stromal cells differentiated rapidly, and no differences were observed between LIF-treated and untreated cultures. Stromal cells produce LIF during *in vitro* culture, and this peaked at 48 h. Freshly collected stromal cells from both day-2 and -4 pregnant mice expressed mRNA for the LIF receptor, and the transcript level was higher in cells isolated on day 4. However, no differences were observed in the relative levels of transcripts in cells from day 2 and day 4 after culture, nor were there differences between the LIF-treated cultures and controls. Therefore, in this study, we have shown that LIF suppresses decidualisation of murine uterine stromal cells in the presence of serum, this is not due to the regulation of PGE₂ secretion by stromal cells.

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Introduction

For implantation of the mammalian embryo, sequential morphological and biochemical changes must occur in both embryo and uterus. The embryo must develop to the blastocyst stage and then hatch prior to attachment to the uterine luminal epithelium. The uterus, in turn, must undergo a differentiation process which makes it receptive, allowing attachment and implantation of the embryo. Following dialogue between the implanting embryo and the endometrium at the implantation site, the embryo can adhere to the luminal surface of the lining epithelium and implant into the uterus. The changes in the uterus are regulated by ovarian steroids and occur both in the luminal epithelium and the underlying stromal cells. Fibroblastic cells of the latter undergo extensive proliferation and then differentiation, a process referred to as decidualisation (Gambel *et al.* 1985, Weitlauf & Suda-Hartman 1988, Abrahamsohn & Zorn 1993, Zhang

& Croy 1996, Kurita *et al.* 2001). Decidualisation involves uterine stromal cell differentiation and remodelling of the extracellular matrix, as well as a remarkable change in the population and distribution of leukocytes in the uterus. Decidualised stromal cells change their shape from fibroblastic to large, polyploid and multinucleated cells (Ansell *et al.* 1974, Monice *et al.* 2001). Among the most obvious modifications during decidualisation is the increase in alkaline phosphatase (ALP) enzyme activity (Finn & McLaren 1967, Pollard *et al.* 1990) and vascular permeability (Psychoyos 1993). In mice and rats, the content of active ALP increases in the antimesometrial decidualised stromal cells at the time of implantation and then spreads through the stromal layer during pregnancy (Murdoch *et al.* 1978, Johansson & Wide 1994). ALP has frequently been used as a marker of stromal cell decidualisation *in vitro* in the rat (Kennedy *et al.* 1998). Decidual cells also express the intermediate filament protein desmin, which appears first in the primary decidual zone, and then progresses

laterally to the mesometrial stromal cells (Glasser *et al.* 1987). In addition, there are changes in oligosaccharide expression – for instance, in the distribution of sialylated structures, such as α 2–6 sialyl residues recognised by lectin SNA (Jones *et al.* 1996), and stage-specific antigen-3 (SSEA-3) (Gendron *et al.* 1997). More recently, many other molecular changes have also been documented, as in transcription factor HoxA-10 (Benson *et al.* 1996) and various signalling molecules (Ying & Zhao 2000, Paria *et al.* 2001).

Several factors have been shown to be capable of acting as inducers or essential factors for decidualisation, including infusion of oil and polysaccharides into the uterine lumen (Finn 1966), steroids (Pollard *et al.* 1990), prostaglandins (PGs) (Kennedy & Doktorcik 1988), platelet activating factor (PAF) (O'Neill 1985), leukotrienes (Kennedy & Doktorcik 1988), transforming growth factor (TGF)- α (Pollard *et al.* 1990), leukemia inhibitory factor (LIF) (Stewart *et al.* 1992, Chen *et al.* 2000) and heparin-binding epidermal growth factor (HB-EGF) (Paria *et al.* 2001). Although a plethora of molecules have been implicated in implantation and decidualisation, most appear not to be essential, as shown by gene deletion in mice. However, maternal expression of both PGs and LIF appears vital for timely and successful implantation of the murine embryo. The levels of PGs and prostacyclins (particularly PGE₂ and PGI₂) increase in implantation sites compared with the pre-implantation uterus or inter-implantation site (Bany *et al.* 1998, Ushikubi *et al.* 1998, Bany & Kennedy 1999). In addition, local administration of PGs induces decidualisation (Sananes *et al.* 1976, Kennedy 1986), and the use of drugs that inhibit PG synthesis (such as indomethacin (IM)) averts or delays decidualisation (Evans *et al.* 1982). PGs are produced from arachidonic acid by the rate-limiting cyclooxygenase (COX) enzymes. In most species, COX-2 is restricted to the implantation site, where it is expressed in luminal epithelium and stromal cells surrounding the active blastocyst during attachment (Chakraborty *et al.* 1996, Lim *et al.* 1999). Indeed, COX-2-deficient mice exhibit multiple reproductive failures, including defects in ovulation and fertilisation, and reduced or delayed decidualisation (Reese *et al.* 1999, Cheng *et al.* 2002).

LIF is a pleiotrophic member of the interleukin 6 (IL-6) cytokine family (Pennica *et al.* 1995, Haines *et al.* 2000). The expression of LIF mRNA and protein peaks first during ovulation and then on day 4 of pregnancy prior to embryo attachment (Bhatt *et al.* 1991, Shen & Leder 2000). LIF acts on its target cells by binding to a low-affinity receptor (LIF-R β) which dimerises with gp130, a protein common to the receptors for other members of the IL-6 family, thus transforming to a high-affinity form for signal transduction (Gearing *et al.* 1992, Song *et al.* 2000). In LIF-null mice, the uterus does not decidualise and blastocysts fail to implant. However, pregnancies do result in LIF^{-/-} mothers after infusion or

injection of exogenous LIF, and LIF^{-/-} embryos can implant after transfer into wild-type pseudopregnant recipient uteri (Stewart *et al.* 1992, Chen *et al.* 2000). Furthermore, by *in situ* hybridisation, it has recently been shown that COX-2 mRNA is aberrantly expressed in LIF^{-/-} mice during implantation (Song *et al.* 2000). The molecular mechanism by which LIF exerts its effect and regulates the implantation process and its interaction with other factors involved in decidualisation and implantation has yet to be clarified.

Uterine stromal cells have been isolated and cultured successfully in a number of species (Glasser & McCormack 1979, Satyaswaroop *et al.* 1979, Siegfried *et al.* 1984, Inaba *et al.* 1988, Arnold *et al.* 2001). With a well-defined culture system, it is possible to understand the characteristics and physiological capabilities of primary cells and their interaction. In the present study, isolated cultured stromal cells underwent decidualisation as assessed morphologically and from immunohistochemical staining for decidual markers and quantitative measurement of ALP activity. The main target for LIF in its regulation of implantation has been suggested to be the luminal epithelium (Chen *et al.* 2000). However, the direct effect of LIF on stromal cells has not been examined. We hypothesised that LIF might directly stimulate decidualisation in stromal cells rather than indirectly via signals from the luminal epithelium. Therefore, the effect of LIF and PGE₂ and their inhibitors on murine stromal cells was examined with ALP as a quantitative measure of decidualisation. Contrary to our expectation, we found that LIF had an inhibitory effect on decidual differentiation as measured by ALP activity, and that this was not the result of suppression of PGE₂ secretion.

Materials and Methods

Animals

Three-week-old MF1 female mice (Harlan Olac, Bicester, UK) were maintained under a controlled photoperiod of 12-h light:12-h dark with food and water supplied *ad libitum*. The mice were induced to ovulate by an intraperitoneal injection of a single dose of 5 IU PMSG (Intervet, Milton Keynes, UK) followed by a single injection of 5 IU HCG (Intervet) 48 h later. Mating was confirmed by the observation of a vaginal plug the following morning (day 1 of pregnancy). The animals were killed by cervical dislocation on either day 2 of pregnancy (48 h after HCG injection) or on day 4. The uterine horns were dissected and trimmed of excess fat and mesentery tissues before transfer into Hanks' balanced salt solution (HBSS; Invitrogen, Paisley, UK).

Chemicals

All of the chemicals were from Sigma (Poole, UK) unless otherwise stated. Primary antibodies and lectins were as

follows: polyclonal rabbit antimouse desmin (1:100), monoclonal rat antimouse SSEA-3 (Hybridoma bank; 1:20), mouse monoclonal immunoglobulin (Ig) M HOO1 (from Dr A Lundblad, University of Lund, Sweden) (Kimber *et al.* 1988) and digoxigenin-labelled *Sambucus nigra* lectin (SNA: Boehringer Mannheim, Germany; 1:50). Fluorescein isothiocyanate (FITC)-conjugated anti-rat or rabbit IgGs (Jackson Labs, Pennsylvania, PA, USA) were used as secondary antibodies at a dilution of 1 in 125. FITC-labelled antidigoxigenin (Boehringer) was used at a dilution of 1:250. Normal goat serum (NGS) was used at a dilution of 1:20 for blocking non-specific binding.

Mouse LIF used as the standard in ELISA was purchased from Chemicon (Chandlers Ford, Hants, UK), and LIF used in culture was a gift from Dr Ann Vernallis (Aston University, Birmingham, UK), and its activity was calibrated by the proliferation response of BAF cells (gift of Dr Ann Vernallis). The LIF inhibitor (LIF 05) that antagonises LIF activation of its receptor was reported previously (Vernallis *et al.* 1997) and used at 10 times the added LIF concentration, or 50 ng/ml, in the absence of LIF. PGE₂ and IM were diluted in culture medium from 1 mg/ml and 0.1 M stocks (respectively) in absolute ethanol. Absolute ethanol was added at equivalent dilution to control cultures in each case.

Isolation and culture of stromal cells

Uterine stromal cells were isolated by a modification of the method established by McCormack and Glasser (1980) and cultured. Briefly, fat-trimmed uteri were cut longitudinally to expose the lumen. They were then placed in the first trypsin dissociation solution (0.5% type II bovine trypsin and 0.165% pancreatin in HBSS (Invitrogen) for 1 h at 4 °C followed by 1 h at room temperature. The medium was then removed (with detached luminal epithelial plaques) and discarded, and the remaining uterine tissue washed and vortexed in ice-cold HBSS. This step was followed by two quick washes in cold HBSS containing 1 µg/ml DNase, 5 µl/ml of 10 mM MgCl₂ and 1% heat inactivated fetal calf serum (HIFCS) (Invitrogen) followed by rapid washing with cold HBSS. At this stage, 10 glass beads were added, together with the second trypsin dissociation solution (0.05% trypsin and 0.02% EDTA (BDH) in HBSS). The tube was vortexed twice during a 20-min incubation at 37 °C and then incubated for 20 min at room temperature with vortexing every 5 min. The content of the tube was then passed through a 70 µm gauze filter (Falcon), and the enzymatic digestion was stopped by adding trypsin inhibitor solution (2% soybean trypsin inhibitor in HBSS) after filtration. The cell suspension was then centrifuged at 400 *g* for 10 min at 4 °C. The pellet was washed in stromal cell culture medium: 1:1 mixture of DMEM and Ham's F12 medium (Invitrogen) supplemented with 1.2 g/l of sodium bicarbonate, 100 IU/ml penicillin streptomycin (Invitrogen)

and 2% HIFCS (Invitrogen), and centrifuged for 10 min at 4 °C. The same batch of serum was used throughout the entire study. The pellet was resuspended in stromal cell culture medium, and live cells were assessed by trypan blue exclusion with a Neubauer haemocytometer. Isolated stromal cells were cultured in 24-well culture dishes (Nunc, Paisley, UK) at 1.5×10^5 cells/ml in a humid atmosphere of 5% CO₂ in air at 37 °C. The culture medium was renewed after 24 h and then every 24 or 48 h. Evaluation was undertaken on a minimum of three cultures in each case.

Immunocytochemistry for decidual markers on cultured stromal cells

Stromal cells were cultured on 13 mm sterile glass cover slips (BDH) in 24-well culture dishes. At 24-h intervals, the culture medium was removed from the cover slips, which were quickly washed with cold PBS, fixed for 5 min in cold acetone (4 °C) and stored at -20 °C until required for immunocytochemistry. This was performed with a 45-min blocking step with 1:20 normal goat serum in PBS containing 1% BSA and 0.05% sodium azide, preceding the primary (overnight at 4 °C) and secondary (90 min at room temperature) antibody. Cover slips were then washed and mounted on glass slides with Gelvatol and examined under a Leica epifluorescence microscope (Leica UK). The intensity of staining was scored and ranged from (+++) for maximum to (---) for no staining.

Measurement of total protein concentration and alkaline phosphatase activity

The cells were lysed in the wells in 250 µl 0.25% deoxycholate. The lysates of three wells were pooled in Eppendorf tubes and stored at -20 °C until use for ALP activity determination and protein assay. Samples for basal activity (T=0) determination were taken before seeding. Freshly isolated cells from three wells were transferred to an Eppendorf tube and centrifuged at 400 *g* for 5 min. The medium was then removed and the pellet resuspended in 750 µl deoxycholate. Samples were processed for total protein determination by the BCA method (Pierce), with BSA (Pierce) as standard and saline deoxycholate solution (250 µl 0.9% saline plus 500 µl deoxycholate) as blank in a 96-well assay plate (Nunc). The colour was read with a microplate reader (Dynatech, MR500) at 570 nm. A standard curve was plotted by using serial dilutions of BSA in saline. A volume of 200 µl of BCA reagent was added to 20 µl of samples and standard solutions, according to the manufacturer's instructions. The activity of ALP enzyme was assessed with the Sigma kit 204 in a 96-well assay plate with a microplate reader. The method is based on the ability of the enzyme to convert *p*-nitrophenyl phosphate to *p*-nitrophenol, which in alkaline solution presents as

a yellow colour and can be read at 410 nm. A standard curve was plotted by serial dilution of the standard. The concentration of ALP in each sample was measured by logarithmic conversion of optical density to absorbance ratio. The activity of ALP enzyme was expressed as ng of product/30 min per mg of total protein.

Cell proliferation assay

Cells were cultured as above and harvested at 24-h intervals until 96 h of culture. The cells were removed with warm (37 °C) trypsin/EDTA ((0.05% trypsin and 1 mM EDTA (BDH)) at 37 °C for 5 min, followed by gentle pipetting and incubation for 10 min at room temperature. The cell suspension was placed in 300 µl HBSS containing 20% HIFCS. Cells were centrifuged and resuspended in 1 ml of fresh HBSS. A volume of 10 µl cell suspension was mixed with an equal volume of trypan blue, and live cells that excluded the dye were counted in a Neubauer chamber.

PGE RIA

The concentration of PGE₂ was measured in the culture media by Sigma RIA, and standards (0–100 pg/ml) were prepared in RIA buffer (0.01 M PBS, pH 7.4, containing 0.1% BSA and 0.1% sodium azide). Volumes of 100 µl sample or standards and 500 µl antibody working solution were added to 1.5 ml Eppendorf tubes. These were vortexed, incubated for 30 min at 4 °C, and then ³H-labelled PGE (Amersham), diluted in RIA buffer to give 6000 c.p.m. in 700 µl, was added. The tubes were vortexed again and incubated for 1 h at 4 °C, and then 200 µl cold dextran-coated charcoal suspension (0.1% dextran, 1% activated charcoal (100–400 mesh) in RIA buffer) was added. This mixture was shaken for 10 min at 4 °C. Finally, the tubes were centrifuged at 800 g for 15 min at 4 °C, and the supernatants were transferred into scintillation vials with 4 ml of scintillation cocktail (Optiphase Hisafe 2, Wallac). The amount of radioactivity in all tubes was measured with a beta-counter (Wallac-M1214) and the sample concentration extrapolated from the standard curve. The values were considered reliable only in the logit interval of ± 2.2 when the unlabelled molecules displace 10–90% of maximum radioactivity bound (Granstrom & Kindahl 1983).

Isolation of total RNA

Total RNA was isolated from both freshly collected stromal and epithelial cells on days 2 and 4 of pregnancy, as well as from stromal cells cultured between 24 and 96 h in the presence or absence of LIF. The latter cells were detached from the wells with a cell scraper (Corning), and the cell suspensions from 10 wells were pooled and centrifuged at 1000 g for 5 min. The supernatant

culture medium was removed, and the pellet was stored in liquid nitrogen. RNA was isolated from all samples with the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. Lysis buffer was added to the cells, and the contents were repeatedly passed through 19 gauge needles, and then centrifuged through Quiashredder membranes (Qiagen) for complete lysis. The concentration of total RNA was measured with a Genequant (Amersham Bioscience, Amersham, UK) spectrophotometer.

RT-PCR for the LIF receptor

A one-step RT-PCR kit (Qiagen) was used according to the manufacturer's instructions for RT and amplification of a 350 bp product with the primers, 5'CTTCGATCC TCAACACAGAGC3' (forward) and 5'TGGTTAGTG CACCCATAGAGG3' (reverse), designed using the sequence of *Mus musculus* LIF receptor mRNA and the Primer 3 program (accession number: NM_013584.1). An amount of 1 µg RNA was used for reverse transcription and PCR over 30 cycles with TM of 60 °C and 5-min extension. For experiments where LIF receptor mRNA transcripts were compared between different groups, the tubes were removed from the cyclor (Eppendorf) every two cycles after cycle 18. Extension was then continued in another machine. β-Actin primers (5'AAA CTG GAA CGG TGA AGG C3' (forward) and 5'CCT GGG CCA TTC AGA AAT TA 3' (reverse)), amplifying a 344 bp product, were used for normalisation, with the cycle number at which β-actin was first detected used to compare cDNA quantities from different samples. The set of amplification cycles used was determined to be in the linear range. A volume of 10 µl product or standards (Roche) mixed with 2 µl of tracking dye was loaded on a 2% agarose (Amersham) gel (containing 2% ethidium bromide) and run at 100 mAmp for 1 h in a BioRad electrophoresis tank. For the negative control, distilled water replaced RNA. A BAF cell line (a gift from Dr Ann Vernallis) that expresses LIF receptor was used as positive control. The bands were visualised and photographed on a transilluminator connected to a Kodak camera.

ELISA for LIF production by stromal cells

Capture and detection antibodies and standards (both hLIF and mLIF) for enzyme-linked immunoassay of human LIF were purchased from R & D Systems, Abingdon, Oxon, UK, and the ELISA was calibrated for use with murine LIF. The assay was performed according to the manufacturer's instructions on Maxisorb 96-well dishes (Nunc). Briefly, assay dishes were coated with 100 µl of capture antibody (4 ng/ml) overnight at room temperature (RT) and the wells were then washed three times with PBS washing buffer. Non-specific binding was blocked with PBS containing 1% BSA for 1 h at RT. The blocking

solution was removed, and 100 µl of either Microcon 100 concentrated (Centricon: Millipore, USA) culture medium or serially diluted standards were added to wells. The dish was incubated for 2 h at RT and washed again, and 100 µl of detection antibody (biotinylated LIF antibody) was added to each well and incubated for 2 h at RT. After washing, 100 µl of peroxidase-conjugated streptavidin HRP (Zymed, 1:200 in 1% BSA in PBS) was added to each well to induce a colour reaction. The reaction was stopped by adding 50 µl of 1 M H₂SO₄. With a plate reader (Dynatech), a logarithmic linear curve was plotted for the ratio of optical density to concentration of standards, and concentrations of LIF in the samples were read from the standard curve.

Statistical analysis

Data are presented as mean ± S.E.M. Statistical analysis was performed with the SPSS program to carry out a two-way analysis of variance on the data. A *post hoc* test was then used to analyse the difference between control and treatments. Tukey's test was also used to reveal the differences between each treatment.

Results

Murine stromal culture and decidualisation *in vitro*

Initially, stromal cells were isolated from day 2 pregnant mice, cultured in the presence of 2%, 5% or 10% HIFCS for up to 96 h, and used for assessment of decidualisation by quantitative measurement of ALP in cell lysates together with other qualitative markers. In all cases, cells with the expected appearance of fully mature decidual cells (large size, frequently binucleate, carrying processes and with a spread appearance) were detected and the frequency of these cells increased with time in culture in 2% and 5% serum. In 10% serum, the number of cells with this appearance was greatly reduced. ALP activity generally increased with time in culture up to 48–72 h, after which it plateaued or decreased. Very low levels of ALP activity were detected in 10% serum, confirming the low frequency of cells with a decidualised appearance (Fig. 1). To minimise the influence of serum factors, 2% HIFCS was used for the later experiments. Decidualisation of stromal cells derived from day 2 and day 4 pregnant mice was compared, using a range of markers. No differences were found between cells from these two days over the period of culture (data not shown). Therefore, stromal cells isolated from day 2 of pregnancy were used for the remainder of the work. Binucleate and giant cells were detected even at 24 h, but these increased at 48 and 72 h of culture (Fig. 2). The results of immunocytochemistry for the decidual markers desmin, ALP, SSEA-3 and SNA are shown in Fig. 2 and summarised in Table 1. Cells stained for decidual markers even after 24 h, but staining

Figure 1 Decidualisation of murine endometrial stromal cells cultured *in vitro*. Stromal cells were isolated and cultured in the presence of 2%, 5% and 10% FCS. Quantitative measurement of ALP activity in cell lysates collected at daily intervals was used to assess decidualisation. ALP activity was significantly higher in stromal cell cultures supplemented with 2% FCS at 48 h ($P < 0.01$) and 72 h ($P < 0.01$) than with the 10% FCS treatment. ** $P < 0.01$. The values represent average ± S.E.M. of three separate replications.

increased with time in culture. The percentages of positive cells for ALP staining were at 24 h: 76%, 48 h: 82% and 72 h: 88% (over three culture experiments). Overall, desmin and ALP staining increased with time in culture, being maximal after 72–96 h. SSEA-3 was detected after 48 h in culture and then increased in intensity at later times. In contrast, SNA lectin binding decreased with time in culture. Cells stained with epithelium-specific antibody markers (H001 and H004) (Kimber *et al.* 1988) were less than 2% of cells by 48 h under these conditions (Fig. 1).

Quantitation of stromal decidualisation response and the effect of PGE₂

To establish the responsiveness of the murine decidualisation model to PGs, the effect of PGE₂ at 1, 10 and 100 ng/ml on stromal cell decidualisation was assessed by measuring ALP activity in cell lysates. PGE₂ had maximum effect at 10 ng/ml, increasing ALP activity after 48 h relative to the control ($P < 0.01$) and after 48 and 72 h relative to 1 ng/ml ($P < 0.05$) and 100 ng/ml ($P < 0.01$) (Fig. 3). The lack of a dramatic effect of added PGE₂ was predictable since it is produced by stromal cells. Therefore, the effect of IM, an inhibitor of PG synthesis, on decidualisation was examined. Stromal cells were treated without (control) or with three concentrations (10^{-4} , 10^{-5} and 10^{-6} M) of IM in the presence of 2% HIFCS, and the activity of ALP was measured (Fig. 4). There was a significant increase in the concentration of ALP in the control group compared with IM-treated cells at all concentrations. In 10^{-4} M IM-treated cultures, a significant reduction in ALP activity was observed at 48 and 72 h ($P < 0.01$ and $P < 0.001$ respectively) compared

Figure 2 Decidualisation of murine stromal cells cultured *in vitro*. Stromal cells were cultured with 2% FCS and assessed for decidualisation, using the increase in giant multinucleate cell (A–D) enzymatic reaction for ALP and immunofluorescence staining for decidual markers, desmin, and SSEA-3 and SNA binding. The intensity of staining and number of decidualised cells increased with time in culture for ALP (E–H), desmin (I–L) and SSEA-3 (Q–T). In contrast, SNA staining was reduced during culture (M–P). Staining for H001 was used to assess the level of contamination with luminal epithelium cells (U–Y).

Table 1 Summary of changes in the intensity of staining for decidual markers. The level of staining was scored as — — — for no staining and + (low), ++ (high), +++ (intense)

Decidual marker	24 h	48 h	72 h	96 h
ALP	+	++	+++	+++
Desmin	+	++	++	+++
SNA	+++	++	++	+
Desmin	— — —	+	+++	+++

with the control group. In 10^{-6} M-treated cultures, a significant reduction in ALP activity was detected at 24 h ($P < 0.05$) and 72 h ($P < 0.001$) in comparison to the control group. Maximum decrease in ALP activity was observed for 10^{-5} M IM-treated cultures at 48 h compared with the control ($P < 0.001$) and other concentrations of IM used (10^{-4} M, $P < 0.05$; 10^{-6} M, $P < 0.01$). At 72 h, there was a significant decrease in the activity of ALP in all IM-treated cultures compared with the controls ($P < 0.001$).

Effects of LIF on proliferation and decidualisation of murine stromal cells

To determine the effect of LIF on stromal cells, we cultured them in the absence (control) or presence of three concentrations of LIF (0.5, 5 and 50 ng/ml) for up to 72 h. Proliferation of stromal cells was measured by counting the number of live cells which excluded trypan blue. Overall, cell numbers increased up to 48 h and decreased thereafter. The number of cells in LIF-containing medium was significantly increased in 5 ng/ml LIF-treated ($P < 0.01$) and decreased in 0.5 ng/ml LIF-treated cultures ($P < 0.01$)

Figure 3 Effect of PGE_2 on decidualisation of murine stromal cells cultured *in vitro*. PGE_2 was added at 1, 10 and 100 ng/ml during culture, and ALP activity was measured in cell lysates. PGE_2 had maximum effect at 10 ng/ml PGE_2 , increasing ALP activity after 48 h ($P < 0.01$) relative to control, and after 48 and 72 h relative to 1 ng/ml ($P < 0.05$) and 100 ng/ml ($P < 0.01$). * $P < 0.05$, ** $P < 0.01$. The values represent average $\pm$ S.E.M. of three separate replications.

Figure 4 Effect of indomethacin (IM) on decidualisation of murine stromal cells cultured *in vitro*. Stromal cells were cultured in the presence of three concentrations of 10^{-4} , 10^{-5} and 10^{-6} M IM in the presence of 2% HIFCS, and the activity of ALP was measured. IM at all concentrations significantly decreased ALP activity. Maximum decrease in ALP activity was observed for 10^{-5} M IM-treated cultures at 48 and 72 h compared with the control ($P < 0.001$) and at 48 h compared with other concentrations of IM used (10^{-4} M, $P < 0.05$; 10^{-6} M, $P < 0.01$). Significant decreases were also observed in 10^{-4} M (48 h ($P < 0.01$), 72 h ($P < 0.001$)), and 10^{-6} M (24 h ($P < 0.05$), 72 h ($P < 0.001$))-treated cultures relative to control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. The values represent average $\pm$ S.E.M. of three separate replications.

compared with the control at 24 h (Fig. 5). At 48 h, cell numbers were greater in the 0.5 ng/ml group than the control ($P < 0.01$). At 72 h, no differences were observed between control and LIF-treated cells.

We also examined the effect of LIF on ALP activity and PGE_2 production in cultured stromal cells (Figs 6 and 7). LIF at all concentrations and time points decreased ALP activity compared with the control, and this inhibition was dose-dependent, with the greatest effect at 5 and 50 ng/ml LIF (Fig. 6). At 72 h, there were no significant differences between 5 and 50 ng/ml LIF or between 0.5 ng/ml and the control. Based on these results, the concentration of 5 ng/ml LIF was used for the later experiments. When PGE_2 was assessed, a significant increase in PGE_2 levels was found with time in culture in all LIF-treated cultures and controls (Fig. 7). However, PGE_2 production was not significantly different between LIF-treated groups and control or between different LIF concentrations. Thus, LIF did not stimulate or decrease PGE_2 production in these cells *in vitro*. The effect of LIF and LIF inhibitor (LIF05) on ALP levels was assessed in stromal cells cultured in the absence (control) or presence of 5 ng/ml LIF, 50 ng/ml inhibitor and 5 ng/ml LIF plus 50 ng/ml inhibitor (Fig. 8). LIF inhibitor significantly increased ALP concentration at both time points compared with LIF-treated cells ($P < 0.05$). In addition, there were significant differences between cultures in LIF alone and LIF plus LIF05 ($P < 0.05$). Again, LIF significantly reduced ALP

Figure 5 Effects of LIF on the cell numbers for murine stromal cells cultured *in vitro*. At 24 h, cell numbers were significantly lower in 0.5 ng/ml LIF-treated cultures and significantly higher in 5 ng/ml LIF-treated cultures relative to the control (** $P < 0.01$). No difference was observed between 50 ng/ml LIF-treated cultures and controls. At 48 h, cell numbers were significantly higher only in 0.5 ng/ml-treated cultures (** $P < 0.01$). No significant differences were observed between the other LIF-treated groups and controls. At 72 h, no differences were observed in any group compared with controls. Cell number is recorded relative to control indicated by the line at 1 on bar graph.

activity at 48 h ($P < 0.01$) and 72 h ($P < 0.05$) compared with the control. There were no significant differences between control and LIF05 or between control and LIF plus LIF05 at either time point. These observations suggested that LIF inhibited ALP activity in 2% serum-containing medium. Therefore, because of the possible compounding effect of serum in the medium, stromal cells were cultured for 24 h with 2% FCS, and then the serum-containing medium was removed. They were divided into four treatment groups, without FCS: no additive (control); 5 ng/ml LIF; 50 ng/ml inhibitor and 5 ng/ml LIF plus 50 ng/ml inhibitor in serum-free conditions. The data were analysed for differences in activity of ALP at 48 h of culture (Fig. 9). There were no significant differences in ALP activity between any of the groups in the absence of serum.

Production of LIF by stromal cells and influence of LIF on its receptor

We asked whether stromal cells make LIF *in vitro*. Production of LIF by the cells cultured up to 96 h was measured by ELISA of culture medium (Fig. 10). The value for cell-free culture medium subtracted to exclude any LIF in the added medium ((LIF) = 0.005 ng/ml in culture medium plus 2% serum). LIF production increased between 0–24 and 24–48 h of culture, and then produc-

tion plateaued at 2.5 ng/ml at 24 h/mg protein, or gradually decreased up to 96 h of culture. Thus, in a 24-h period, the cells were producing between 1.6 and 2.5 ng/ml LIF/mg protein. With an average cell protein of 0.15 mg/well at 24 h, this would give 0.24 ng LIF/well per 24 h at 24 h and with an average cell protein of 0.35 mg/well at 48 h, 0.88 ng LIF/well per 24 h at 48 h, accounting for the difference between the control and the inhibitor alone in the experiments above.

To assess the effect of culture *per se* or the addition of LIF on expression of LIF receptor, the transcript for LIF receptor was examined by a semi-quantitative method. cDNA was normalised, using β -actin. In cDNA isolated from freshly collected day 4 stromal cells, a band was first detected after 20 cycles, while in freshly isolated day 2 cells it appeared after 26 cycles (Fig. 11A). When LIF-R β was assessed in cDNA from cultured cells, it was found that the band was first detected at 20 cycles irrespective of the number of days in culture. In addition, no differences in the cycle number at which a band could first be detected were found between day 2 and day 4 stromal cells after 24 h or more in culture (Fig. 11B and C). Thus, while *in vivo* there are differences in transcript abundance between day 2 and day 4 of pregnancy, these differences disappear after culture. For cultured cells, there was no difference between control and any dose of LIF on the cycle number at which LIF-R β was detected (Fig. 11B and C).

Figure 6 Effects of LIF on ALP activity in murine stromal cells cultured *in vitro*. Stromal cells were cultured in the absence (control) or presence of 0.5, 5 and 50 ng/ml LIF. The activity of ALP measured in cell lysates was significantly reduced in all LIF-treated groups in comparison with the control in each time point. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. The values represent average $\pm$ S.E.M. of six separate experiments.

Discussion

We employed an *in vitro* culture system for murine uterine stromal cells based on that employed for the rat (Inaba *et al.*

1988). In these cultures, mouse stromal cells spontaneously decidualise by a number of criteria, including increased activity of ALP, which was used as a quantitative measure of decidualisation. In establishing the system, the effects of

Figure 7 Effects of LIF on production of PGE_2 by cultured endometrial murine stromal cells over 24-h periods up to 72 h in culture, expressed as ng PGE_2 /ml per μg protein. Stromal cells were cultured in the absence (control) or presence of 0.5, 5 and 50 ng/ml LIF. Concentration of PGE_2 in culture media as measured by RIA increased significantly with time in culture. However, there were no significant differences in PGE_2 levels at different LIF concentrations or between LIF and control groups. The data represent average $\pm$ S.E.M. in three separate experiments.

Figure 8 Effects of LIF inhibitor on ALP activity in cultured murine endometrial stromal cells. LIF inhibitor significantly increased ALP concentration at all time points compared with LIF-treated cells ($*P < 0.05$). There was also a significant difference between LIF alone and in the presence of inhibitor, which had a slight stimulatory effect at 48 h ($*P < 0.05$). There were no significant differences between control and LIF inhibitor or between control and LIF plus inhibitor at either time point.

three levels of FCS were tested. In 2% FCS, ALP activity in cell lysates showed a rapid increase up to 48 h and then declined. The increase in ALP was slower in cultures

containing 5% serum, and ALP activity was suppressed in cultures with 10% serum. ALP is a glycosylated, membrane-bound metalloenzyme, and its activity has

Figure 9 Effects of LIF and its inhibitor on ALP activity in serum-free cultures. Murine endometrial stromal cells were cultured for an initial period of 24 h with 2% FCS and then divided into four treatment groups: no additive, 5 ng/ml LIF, 50 ng/ml inhibitor and 5 ng/ml LIF plus 50 ng/ml inhibitor in serum-free conditions. The histogram compares mean $\pm$ S.E.M. of ALP activity at the 48-h time point in cell lysates from three different experiments.

Figure 10 Production of LIF by cultured murine endometrial stromal cells. Uterine stromal cells were isolated and cultured for up to 96 h with 2% FCS. The concentration of LIF in culture media was measured by ELISA and subtracted from the concentration of LIF in FCS. The values represent mean $\pm$ S.E.M. from three separate experiments.

been used extensively as a decidual marker for *in vivo* studies on rat and mouse uteri (Buxton & Murdoch 1980, Kennedy & Ross 1993, Milligan *et al.* 1995) and *in vitro* culture of rat uterine stromal cells (Yee & Kennedy 1991, Kennedy & Ross 1997). Johanson and Wide (1994) found an increase in ALP mRNA during pregnancy that correlated with the increase in ALP activity. The results of the present study confirm that the degree of cellular differentiation is dependent on the concentration of serum in culture. At higher levels of FCS, stromal cells had higher proliferation rates, but the percentage of ALP-positive cells was 3–10% higher for 2% serum than 10% serum supplemented cultures (data not shown), and the ALP activity was up to seven times greater. Attempts to grow cells in less than 2% serum gave inconsistent results and, in our hands, generally did not lead to established primary cultures. This was not due to differences in the serum batch, since we used the same batch throughout this study. Serum contains growth factors, hormones, macromolecules and other components and is added to cell cultures as a survival agent or stimulator of proliferation. It has been reported that FCS contains serum antiapoptotic factor (SAF), which regulates Fas-mediated apoptosis (Kikuchi *et al.* 1995). We found that 2% serum stimulated adequate cell proliferation while still allowing good differentiation of decidualised cells.

Decidualisation of cultured stromal cells was confirmed by their expression of ALP, desmin, SNA-ligand and SSEA-3. By histochemical staining, ALP has been shown in luminal epithelium from day 2 of pregnancy and decidualising stromal cells have been shown from day 6 in

mouse uterus (Bucci & Murphy 1995). In the present study, the number of ALP-positive cells increased with time in culture. The intermediate filament protein desmin has also been used as a marker of decidualisation (Glasser & Julian 1986), and this molecule also showed increased expression as culture progressed. There are also modifications in the surface glycans of rat and mouse uterine stromal cells during the decidual remodelling of extracellular matrix (Jones *et al.* 1993). For instance, a reduction in SNA binding in the murine primary decidual zone was reported on day 6 of pregnancy (Jones *et al.* 1996). We observed intense staining for SNA lectin in the stroma on day 2 of pregnancy, but after implantation on day 5 the staining was reduced in the subepithelial stroma adjacent to the embryo (data not shown). In the present study, SNA lectin binding was high at 24 h and decreased thereafter, an observation which is in keeping with the previous observations. SSEA-3 is a carbohydrate-antigen present on the zygote, in early cleavage stages and in post-implantation embryos (Shevinsky *et al.* 1982). It is expressed in mouse uterine decidual cells after day 6 of pregnancy (Fox *et al.* 1984). For this epitope, the intensity of staining was found to increase with time of culture. Thus, expression of several decidual markers is similar *in vitro* to that observed *in vivo*, confirming the utility of the culture system.

PGs are known to contribute to various cellular functions, including proliferation and decidualisation (Rankin *et al.* 1979). Indeed, rodent stromal cells produce endogenous PGs that stimulate ALP activity during culture (Yee & Kennedy 1991). After endogenous PG synthesis was

Figure 11 Expression of mRNA for LIF receptor in murine endometrial stromal cells. Total RNA was extracted from either of freshly collected stromal cells (A) or cells cultured from day-2 (B) and day-4 (C) pregnant mice for 24–72 h. Expression of mRNA for LIF receptor was assessed by semi-quantitative RT-PCR on 1 µg of RNA in each lane at different cycles. In freshly collected day-4 stromal cells, the first band was detected after 20 cycles while in day-2 cells it appeared after 26 cycles (A), but there were no differences between cultured stromal cells in the control groups and LIF-treated cells from either day (B and C).

inhibited by IM, PG infused into the lumen of sensitised rat uteri restored the endometrial vascular permeability response and decidualisation (Kennedy & Lukash 1982, Hamilton & Kennedy 1994). Among various PGs, PGE₂ and prostacyclin have been considered as primary candidates involved in implantation and decidualisation (Kennedy 1985, Daniel & Kennedy 1987, Kennedy & Ross 1993, Pakrasi 1997, Lim *et al.* 1999, Papay & Kennedy 2000). PGE₂ receptor subtypes show tissue specific expression in the peri-implantation mouse uterus (Lim & Dey 1997, Yang *et al.* 1997), and PGs and mRNAs and proteins for receptors and enzymes involved in PG synthesis are highly expressed in the implantation site and decidual cells in mouse (Chakraborty *et al.* 1996,

Pakrasi 1997, Yang *et al.* 1997, Ni *et al.* 2002b). PGE₂ may exert its effect through upregulation of cAMP activity (Yee & Kennedy 1991). Alternatively, it has been suggested that the major mediator of decidualisation in mice is prostacyclin (PGI₂), acting via the nuclear hormone receptor PPARδ (Lim *et al.* 1999). PGE₂ also enhances decidualisation of human endometrial cells cultured in the presence of progesterone (Frank *et al.* 1994). In the present study, ALP activity increased in cultures supplemented with PGE₂ compared with the control and with time in culture. An amount of 10 ng/ml PGE₂ significantly stimulated ALP activity compared with 1 and 100 ng/ml PGE₂, suggesting that superphysiological amounts of PGE₂ may lead to receptor downregulation. Inclusion of IM, an

inhibitor of PG synthesis, reduced ALP activity at all concentration used, and addition of PGE₂ reversed this effect in a dose-dependent manner. Similar results have been reported in rat stromal cells (Kennedy & Ross 1993, 1997), and such PG-mediated stimulation of ALP has been reported in osteoblasts (Sisk *et al.* 2001).

Evidence for the essential role of LIF in implantation of the mouse embryo comes from the infertility of LIF-null female mice together with the tightly regulated transient expression just before implantation (Bhatt *et al.* 1991, Stewart *et al.* 1992, Stewart & Cullinan 1997). Furthermore, decidualisation has been shown to be severely defective in LIF-null females (Chen *et al.* 2000). Since LIF-R β is expressed at the highest levels by the luminal epithelium, it is inferred that LIF exerts its major effect on decidualisation via signals transduced by that tissue (Cheng *et al.* 2002). In the present study, we examined the direct effect of LIF on stromal cells *in vitro*. Although these cells did not comprise a pure population, our own observations and those of others (Jacobs & Carson 1993) indicate that in this type of culture less than 2% cells are epithelial and less than 1% consist of leukocytes by 48 h of culture. Addition of LIF to murine stromal cells in culture caused a concentration-dependent inhibition of ALP activity compared with the control. The percentage of ALP-positive cells was also lower for LIF-treated cultures (data not shown). *In vitro*, LIF similarly suppresses ALP activity in osteoblast-like cells (Thiede *et al.* 1988) and rat calvaria cell cultures (Malaval *et al.* 1995). The suppressing effect of LIF on ALP activity is paralleled by a slight stimulatory effect on cell numbers, observed at 24 h in cultures with 5 ng/ml LIF, but only later, at 48 h, in medium with only 0.5 ng/ml added LIF. At 50 ng/ml, it had no influence on cell numbers, due to a threshold effect or possibly because of downregulation of its receptor. However, at 50 ng/ml, LIF is still effective at blocking ALP activity to a level at least equivalent to 5 ng/ml added LIF.

Mouse uterine stromal cells produce LIF during culture, and the amount of LIF produced/24-h period is highest at 24–48 h and then decreases. So we must consider the effects this endogenously produced LIF has on both proliferation and differentiation of stromal cells. At 48 h in 0.5 ng/ml-treated cultures, there is, on average, an extra 0.88 ng/well of endogenously produced LIF present that can stimulate proliferation. The actual concentration is therefore approximately 1.3 ng/ml. This is apparently sufficient to induce equivalent increase in cell numbers by 48 h to that triggered by 5 ng/ml (+0.88 ng/ml produced by the cells) after 24 h of culture. Inhibition of stromal apoptosis by LIF cannot be ruled out.

To further investigate the role of LIF in decidualisation, we used hLIF 05, a mutant form of LIF that competes with LIF for binding to its receptor, which has already been demonstrated to be a powerful antagonist of LIF (Hudson *et al.* 1996). We observed that LIF 05 slightly enhanced

ALP activity compared with control cultures, presumably due to the antagonism of LIF produced by the cells. We also observed that, in serum-free cultures, we could detect no significant difference in ALP activity between LIF-treated and untreated groups or in the presence of the inhibitor. This suggests that LIF may act with serum factors to inhibit ALP. It is notable that the cells cease proliferation and either differentiate or die very quickly in the absence of serum, suggesting very rapid commitment to one or other of these pathways and lack of maintenance of a plastic proliferating population. It is probable that the lack of other factors precludes us from observing an effect of LIF.

LIF acts on cells by binding to its heterodimeric receptor, comprising gp130 and LIF-R β (Gearing *et al.* 1991). By *in situ* hybridisation, LIF-R β mRNA appears to be mainly localised to the luminal epithelium and gp130 mainly to the glandular epithelium on days 3–5 of pregnancy (Cheng *et al.* 2001). However, LIF-R β mRNA is detected strongly in decidua from day 6 of pregnancy, and LIF-R β and gp130 protein have been detected in the stroma on day 5 (Yang *et al.* 1995, Ni *et al.* 2002). Thus, although the initial site of action for LIF on day 4 may be the luminal epithelium (LE), it is clear that LIF can interact with the decidualising stroma, particularly later during the implantation process, and our data indicate that this interaction may be a repressive one.

Relative measurements of transcript level for LIF-R β suggested the presence of greater numbers of transcripts in freshly isolated stromal cells on day 4 than day 2 of pregnancy. However, it is important to remember that freshly isolated cells are a mixed population containing glandular epithelial cells, leukocytes and endothelial cells as well as fibroblastic cells. By 24 and 48 h of culture, stromal cells from days 2 and 4 of pregnancy appeared to have similar levels of transcripts for LIF-R β . This was not influenced by the addition of LIF to the culture medium. It seems that cultured stromal cells retain expression of LIF-R β , and some upregulation may be apparent for stromal cells from day 2 of pregnancy, which are capable of spontaneous decidualisation *in vitro* in the absence of stimulation *in vivo* by nidatory oestrogen. Our results tentatively suggest that the effect of LIF in suppressing decidualisation of stromal cells may be independent of any negative (or positive) feedback regulation on its own receptor mRNA. However, modulation of gp130 mRNA levels or feedback on post-transcriptional events cannot be ruled out.

Overall, we have shown that uterine stromal cells decidualise *in vitro* with a time course dependent on the concentration of serum in culture. LIF reduces decidualisation of mouse uterine stromal cells during culture, and this effect was suppressed by a competitive inhibitor of LIF. *In vitro*, decidualising stromal cells produce LIF, and this reflects the known expression of LIF transcripts by decidual cells *in vivo*. Although it appears that LIF has a

major critical target in the LE which is essential for implantation, we suggest that LIF may also have an important role in spatially restricting the decidual response to the implantation site at days 4–5 of pregnancy, when additional localised embryonic signals, transduced through a LIF-stimulated luminal epithelium, promote decidualisation. Its transient expression and source in uterine glands (Bhatt *et al.* 1991), together with receptor expression by decidua (Ni *et al.* 2002a), is in keeping with such a role. Thus, at the implantation site, as the area of initial decidualisation spreads through the stroma towards the myometrium, from the region adjacent to the implanting embryo, the uterine glands become restricted to the region directly subjacent to the muscle layers. However, in the inter-implantation site (where decidualisation does not occur), they remain dispersed through the stroma. LIF protein may still be present to enact this inhibition after day 4 of pregnancy, since, although LIF mRNA decreases, it is still detectable on day 5 by Northern blotting (Bhatt *et al.* 1991), and LIF protein is reported to be expressed stably through the first 5 days of pregnancy in myometrium (Yang *et al.* 1995), which would also be suitably placed as a source of a decidual restraining factor. Finally, expression of LIF by decidualising stroma itself may act to limit decidualisation by autocrine feedback mechanisms, since we have shown that both the receptor mRNA and LIF protein are produced by decidualised stromal cells *in vitro* and LIF-R β and gp130 are co-expressed in decidua *in vivo* (Ni *et al.* 2002a).

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