Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation

(mouse embryo/uterus/estrogen)

Harshida Bhatt, Lisa J. Brunet, and Colin L. Stewart*

Department of Cell and Developmental Biology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110

Communicated by Herbert Weissbach, September 18, 1991

ABSTRACT We have analyzed the expression of the cytokine leukemia inhibitory factor (LIF) during embryogenesis and in tissues of neonatal and adult mice. The site of the most abundant LIF expression is the uterine endometrial glands, specifically on day 4 of pregnancy. Analysis of LIF expression in pseudopregnant mice and in females undergoing delayed implantation showed that it is under maternal control and that its expression coincides with blastocyst formation and always precedes implantation. These results suggest that a principal function of LIF in vivo may be to regulate the growth and to initiate implantation of blastocysts.

Leukemia inhibitory factor (LIF) or DIA (differentiation inhibitory activity) is a 45- to 55-kDa secreted glycoprotein (1, 2) that has multiple activities on various in vitro culture systems. These include the induction of the acute-phase response in hepatocyte cultures (3), the regulation of the differentiation and proliferation of certain hematopoietic cell lines (4–6), the establishment of the cholinergic phenotype in rat sympathetic neurons (7), the remodeling of bone (8, 9), and the inhibition of embryonic stem cell differentiation in vitro (10, 11). Little is known about the in vivo function(s) of LIF, although mice that have high levels of circulating LIF undergo excessive bone formation, become cachexic, and eventually die (12). The observation that LIF inhibits embryonic stem cell differentiation in vitro also suggests that its role in regulating the growth and development of early mouse embryos, since embryonic stem cells are derived from the inner cell mass of blastocysts. Derivation of embryonic stem cells from the inner cell mass is dependent either on the presence of a fibroblast "feeder" cell layer that secretes LIF or on exogenous LIF added to the culture medium (13–17).

To gain further insights into its function(s) in vivo, we have analyzed the temporal and spatial expression of LIF during embryogenesis in neonatal and adult mice. Our results show that LIF is expressed at low levels in many different tissues, but the highest level of LIF mRNA expression occurs in the endometrial glands of the uterine endometrium. Expression occurs transiently on day 4 of pregnancy (i.e., prior to implantation), and this suggests that one of LIF’s principal functions in vivo may be to regulate blastocyst growth and implantation.

MATERIALS AND METHODS

Isolation of Total RNA from Mouse Tissues. Mice, (C57BL/6J × CBA)F1 hybrids (The Jackson Laboratory) or FVB (Taconic Farms), were sacrificed by cervical dislocation, and various tissues were removed, quick frozen in liquid nitrogen, and stored at −70°C.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Uteri at different stages of preimplantation pregnancy were removed. To ensure that they contained viable embryos, delayed blastocysts, or dead embryos, uteri and oviducts from mice respectively undergoing normal pregnancy, delayed implantation, or pseudopregnancy were flushed (18) to determine that the appropriate embryonic stage could be found that corresponded to the particular time of pregnancy. Any uteri or oviducts not containing the appropriate embryonic stage were discarded.

Total RNA was prepared by homogenizing the tissue in RNAzol B (Cinna/Biotecx Laboratories, Houston), and RNA was extracted according to the manufacturer’s instructions.

Analysis of Total RNA. Total RNA was analyzed for the presence of LIF transcripts by RNA blot hybridization (Northern analysis) and washed under high-stringency conditions (19). RNase protection was performed by using the Ambion RPA procedure (Ambion, Austin, TX) according to the manufacturer’s instructions. The LIF probe for both assays was the 577-base-pair (bp) insert from the cDNA clone LIF Mut 1 (1), labeled by incorporation of [α-32P]dUTP by transcription using the T7 promoter. The mouse β-actin probe, supplied by Ambion, was used as a control. RNA levels were quantified by using a Cliniscan densitometer (Helena Laboratories).

In situ RNA hybridization and histological analysis were performed, essentially under conditions described by Wilkinson et al. (20). For histological analysis, sections were stained with hematoxylin and eosin.

Mice Undergoing Delayed Implantation. Derivation of mice was performed surgically or induced naturally as described by Bergstrom (21). Implantation was initiated in these mice on day 6 or 7 after plugging (days 3 or 4 after ovariectomy) by a single intraperitoneal injection of 0.2 μg of β-estradiol (Sigma) dissolved in ethanol and diluted to the final concentration in corn oil (Mazola). In naturally induced mice, implantation was initiated by removing the suckling pups.

RESULTS

LIF mRNA Expression. Total RNA was isolated from adult and neonatal tissues and from postimplantation embryos starting on day 8 of gestation (day of plug = day 1 of pregnancy). RNAs were analyzed by using either the blot hybridization or RNase protection procedures. The majority of tissues and embryonic stages analyzed, expressed LIF RNA either at low levels or at levels that were undetectable with these techniques. A representative set of examples is shown in Fig. 1, and the results are summarized in Table 1. Detectable levels of LIF expression were found in neonatal skin, neonatal calvaria, the yolk sac of 11- to 16-day gestation embryos, and in the small intestine of adult mice. However, these levels were lower than those found in primary mouse

Abbreviation: LIF, leukemia inhibitory factor.

*To whom reprint requests should be addressed.
embryo fibroblasts (PMEFs), which are known to express and secrete LIF (10). The highest level of LIF mRNA expression was found in uteri isolated from pregnant mice. Since other cytokines such as murine colony-stimulating factor 1 (22, 23) and human interleukin 6 (24) are also expressed in the uterus, we undertook a more detailed analysis of LIF expression in this tissue during pregnancy.

Uteri from mice at different stages of pregnancy were removed, and total RNA was isolated. Uteri and oviducts isolated during the first 5 days after fertilization were flushed prior to RNA preparation to ensure that they contained viable embryos. Uteri from postimplantation stages were dissected free of the conceptus, decidua, and fetal membranes. No LIF transcripts were detected in uteri from mice that were in estrous but had not been mated (see Fig. 4, lane 12). After fertilization there was a slight increase in expression of a 4.2-kilobase (kb) transcript (Fig. 2) on the first day of pregnancy. However, a distinct burst of expression resulting in a 5-fold increase in transcript level (as measured by densitometry) occurred on day 4 of gestation. By day 5, LIF RNA levels had declined to less than that detected on day 1, and this level of expression continued throughout the rest of the pregnancy (Fig. 2).

**In situ Hybridization Analysis of LIF Expression.** To further characterize the pattern of LIF expression described above, longitudinal and sagittal sections were made from uteri during each of the first 8 days of gestation and on day 18 (days 5–8 included sections through the uterus, the decidua, and the embryo). These were subjected to in situ hybridization analysis with LIF sense and antisense probes. With the antisense probe, a strong signal was detected on day 4 of gestation in cells of the endometrial glands located in the uterine endometrium (Fig. 3B). Weaker signals were seen in the endometrial layer lining the uterine lumen on days 1–3, with a slightly stronger signal present on day 1 (data not shown). By day 5 of gestation, when blastocysts had already begun to implant with concomitant formation of decidua, the endometrial glands were degenerating, although some still exhibited a weak signal (data not shown). Analysis of uteri from postimplantation stages, including the decidua and embryo on days 7 and 8 of gestation, did not reveal any sites of detectable LIF mRNA expression. Sense probes failed to produce any signal. These results suggest that as embryos

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Expression level</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMEFs</td>
<td>++</td>
</tr>
<tr>
<td>Days 1–3 pregnant uterus</td>
<td>+</td>
</tr>
<tr>
<td>Day 4 uterus</td>
<td>+++</td>
</tr>
<tr>
<td>Days 5–19 pregnant uterus</td>
<td>+</td>
</tr>
<tr>
<td>Nonpregnant uterus</td>
<td></td>
</tr>
<tr>
<td>Days 8–17 postimplantation embryo</td>
<td></td>
</tr>
<tr>
<td>Fetal yolk sac</td>
<td>±</td>
</tr>
<tr>
<td>Decidua</td>
<td></td>
</tr>
<tr>
<td>Newborn calvaria</td>
<td>+</td>
</tr>
<tr>
<td>Newborn skin</td>
<td>+</td>
</tr>
<tr>
<td>Adult spleen</td>
<td>±</td>
</tr>
<tr>
<td>Bone marrow</td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td>±</td>
</tr>
<tr>
<td>Heart</td>
<td>±</td>
</tr>
<tr>
<td>Lungs</td>
<td>±</td>
</tr>
<tr>
<td>Ovary</td>
<td>±</td>
</tr>
<tr>
<td>Testis</td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
</tr>
<tr>
<td>Intestine</td>
<td>+</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>±</td>
</tr>
<tr>
<td>Brain</td>
<td>±</td>
</tr>
<tr>
<td>Salivary gland</td>
<td></td>
</tr>
</tbody>
</table>

The level of expression has been estimated relative to that in primary mouse embryo fibroblasts (PMEFs), which is marked ++ [see Fig. 1]. A dash indicates no detectable expression. (Assays were performed with a densitometer.)

Fig. 1. RNAse protection analysis of LIF expression in some tissues analyzed from adult and embryonic stages of the mouse. Samples were prepared and analyzed as described. Note the strongest signal was present in the day 4 (D4) uterus with weaker signals detected in primary mouse embryo fibroblasts (PMEF), yolk sac, and small intestine.

Fig. 2. Northern analysis of LIF expression in the uterus during pregnancy. LIF transcripts were detected as a single 4.2-kb band. The day of pregnancy (day 1 = day of plug) is shown at the top. Also shown is the expression of LIF found in primary mouse embryo fibroblast (PMEF) cultures alone or treated with cycloheximide (Cyclo) for 5 hr prior to RNA isolation. As a control for the amount of RNA loaded, the filter was also probed with the constitutively expressed gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The levels of LIF expression in the uterus remain relatively constant throughout pregnancy with a slight increase on day 1 but a marked increase on day 4.
move from the oviduct into the uterus, LIF transcripts begin to accumulate in the endometrial glands. Therefore, the onset of LIF expression in normal pregnancy coincides with overt blastocyst formation and is prior to implantation.

**Maternal Regulation of LIF Expression.** Coordinate onset of LIF expression and development of embryos into blastocysts in the uterus on day 4 suggest that either LIF expression could be induced by a signal(s) originating from the embryo or it could be under maternal control. To distinguish between these two possibilities, we performed RNase protection and *in situ* analysis on uteri taken from pseudopregnant mice (derived by mating females to vasectomized males and subsequently inspected to ensure that no viable embryos were present). Analysis of uterine RNA samples isolated from these animals on days 1–5 of pregnancy again revealed a distinct burst of expression occurring on day 4 (six females were analyzed, and all expressed LIF) (Fig. 4, lanes 8 and 9). These results indicate that expression of LIF mRNA in the uterus is not induced by the presence of viable embryos in the uterine lumen. Rather, it suggests that LIF expression is under maternal control. To further analyze this, we examined the pattern of LIF expression in mice undergoing either experimentally induced or natural occurring delayed implantation.

Experimentally induced delay of implantation is achieved by bilateral ovariectomy in day 3 of gestation with subsequent administration of progesterone (21). Removal of ovaries prior to day 4 is necessary, as it prevents an estrogen surge from occurring on days 3–4 that is necessary for implantation (25). In these delayed mice, embryos develop to the blastocyst stage in the uterine lumen where they remain viable but do not implant, and cell proliferation in the embryo is also reduced (26, 27). Such a delayed state can be maintained for up to 7–10 days after fertilization. A single intraperitoneal injection of estrogen into the mother is sufficient to overcome this delay, resulting in implantation 12–24 hr later.

Natural delayed implantation occurs spontaneously when a female that has just littered is mated again (27, 28). The second generation of embryos develop normally, but the blastocysts enter into a state of delay in the uterine lumen because of the hormonal milieu induced by lactation and suckling by the first litter. Removal of the first litter (and therefore loss of the suckling stimulus) results in the onset of implantation of the second set of embryos.

Analysis of mRNA levels in uteri from 10 mice undergoing experimentally induced delay and 4 mice undergoing naturally induced delay on the day(s) equivalent to days 4–7 postconception, revealed that LIF mRNA expression was either absent or barely detectable (Fig. 4, lanes 1, 6, and 7). As previously described, only uteri containing delayed blastocysts and with no indication of implantation occurring, such as decidual swellings, were used in the analysis. Histological and *in situ* analysis of delayed uteri revealed that endometrial glands were still present but did not express LIF mRNA (data not shown).

Four mice undergoing experimentally induced delay were then given a single injection of β-estradiol (0.2 μg) on day 6 of gestation, and nine mice undergoing a natural delay had their litters removed. Uteri were removed 12–18 hr later, and LIF mRNA levels were determined. In all four mice injected with estrogen, LIF expression was equivalent to that found in normal day 4 gestation uteri. Six of the nine females that had their pups removed also had significantly increased levels of LIF expression (Fig. 4, lanes 2–5). These results reveal that uterine LIF expression is under maternal control, possibly via the direct action of estrogen. The timing of the expression correlates with the onset of blastocyst implantation whether in normally mated, pseudopregnant, or delayed implantation mice.

**DISCUSSION**

To gain further insights into the biological role of LIF, we have analyzed its spatial and temporal distribution during mouse development. Our results show that LIF expression occurs in a relatively wide variety of adult tissues and at different stages of development, usually at very low levels. Higher levels of expression were detected in the small intestine of adult and skin of newborn mice. The significance of these increased levels is obscure, although these tissues do contain stem cell populations, and LIF might be involved in regulating their growth and differentiation.
Expression in postimplantation embryos was either undetectable or occurred at very low levels, which is in broad agreement with previous studies where detectable levels of LIF expression were found in the extraembryonic membranes and placenta (29); the latter probably contained a maternal contribution derived from the uterus. Significant expression was not found in midgestation embryos (days 11–17), which was surprising since relatively high levels of LIF expression occur in primary embryonic fibroblasts derived from these embryonic stages. However, LIF expression can be regulated in cell culture by exogenous factors (30), and it is conceivable that factors present in culture medium may initiate LIF expression with the establishment of fibroblast cultures.

The most abundant levels of LIF expression were found in the uterus, specifically in endometrial glands, on days 4–5 of pregnancy. The timing and location of this expression is intriguing. Since LIF is not expressed in the endometrial glands prior to the onset of implantation or during delayed implantation when endometrial glands are also present but is expressed on day 4 of normal pregnancy when blastocyst formation occurs and also once delay is overcome, the principal function of LIF may be to regulate implantation. The finding that LIF expression is not dependent on the presence of viable embryos and occurs on day 4 in pseudopregnant females indicates that expression is under maternal control, possibly as a direct response to the increase in circulating estrogen levels that occurs on days 3–4 of pregnancy (25). This suggestion is supported from analyzing LIF expression during delayed implantation where, even though viable blastocysts were present in the uterine lumen and endometrial glands were in the endometrium, LIF expression did not occur until delay was broken either by injecting females with estrogen or removing the first litter of pups.

It is not known whether the burst of LIF expression in the uterus results in secreted LIF binding to and acting on endometrial cells of the uterus and/or blastocyst. There are some indications that LIF may act on the embryo since trophectoderm expresses receptors that bind LIF (D. Hilton and R. L. Williams, personal communication). Also, blastocysts cultured in vitro in the presence of LIF hatch from the zona pellucida and attach to the surface of the culture plate at higher frequency than controls (33). Attempts were made to determine whether the presence of LIF would stimulate cultured blastocysts to alter their uptake of radiolabeled nucleotides or amino acids, as has been reported for the action of insulin on blastocysts (31), but no marked effect was detected (data not shown).

It is possible that, like insulin, the burst of LIF expression may also have a direct effect on the inner cell mass of blastocysts, the embryonic source of embryonic stem cells. This would, at first, appear unlikely since the inner cell mass is surrounded by the trophectoderm, a supposedly impermeable barrier. However, recent studies have shown that polar trophectoderm is capable of binding insulin and translocating it across the cell in coated pits where it is then presented to and taken up by the inner cell mass (31). Although there is some evidence to suggest that cultured blastocysts can sometimes synthesize LIF (as detected by polymerase chain reaction analysis) (29, 32), this may have been due to stimulation by exogenous factors as reported (30). Thus, in vivo, the principal source may be endometrial glands secreting it into the uterine lumen.

In conclusion, these studies have shown that prior to the onset of blastocyst implantation in mice, there is a dramatic, but transient burst of LIF expression in endometrial glands of the uterus which is then reduced once the implantation is completed. The coincidence in timing suggests that LIF may be acting in some manner to stimulate the blastocyst, whether directly or indirectly, to implant. In turn, these studies suggest that analysis of LIF expression and other cytokines during pregnancy in other mammals could be of substantial value for improving methods of embryo culture, isolation of embryonic stem cells from other species, and in supporting the growth and development of embryos derived by fertilization in vitro.

We thank Jill McMahon for her excellent advice on the in situ technique, Nick Gough for sending us the LIF cDNA, Paul Waserman for helpful comments, and Sharon Perry for typing the manuscript.

8. Allan, E. H., Hilton, D. J., Brown, M. A., Evely, R. S.,