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In Vivo Effect of Leukemia Inhibitory Factor (LIF) and an Anti-LIF Polyclonal Antibody on Murine Embryo and Fetal Development Following Exposure at the Time of Transcervical Blastocyst Transfer

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ABSTRACT

Leukemia inhibitory factor (LIF) enhances in vitro murine preimplantation development in a time- and dose-dependent fashion. Knockout experiments have demonstrated that endometrial LIF is essential for in vivo murine implantation. We assessed the impact of LIF and an anti-LIF polyclonal antibody (pab) on in vivo development and developed a novel and successful nonsurgical method of embryo transfer for this species, a transcervical blastocyst transfer technique. The objectives of this study were to evaluate the effects of LIF and the anti-LIF pab on 1) implantation, resorption, pregnancy, and viability rates and 2) the overall structural and skeletal development. Two-cell embryos were recovered from superovulated mated donors, cultured to the expanded blastocyst stage, and transferred transcervically into pseudopregnant recipients. Exposure to 5000 U/ml LIF resulted in significant increases in implantation, pregnancy, and viability rates compared with controls. A similar dose of pab produced overall inhibitory effects with a significant decrease in implantation rate. Paradoxically, lower pab doses resulted in significantly increased viability rates. Exposure to LIF had no effect on fetoplacental development. However, pab treatments had variable but significant negative effects on placental length, ossification of the exoccipital bone, and vertebral space width compared with controls. Exposure of murine blastocysts to LIF at the time of transcervical transfer resulted in pronounced positive effects on implantation and pregnancy rates without affecting fetal development. A similar pab dose dramatically reduced implantation and pregnancy rates; at high and low doses, pab produced deleterious effects on placental and skeletal development.

cytokines, implantation

INTRODUCTION

Leukemia inhibitory factor (LIF) is a pleiotropic and multifunctional cytokine with paradoxical biological activities. This compound stimulates differentiation and inhibits cell proliferation in the murine myeloid leukemia cell line M1, whereas in embryonic stem cells it inhibits differentiation and promotes proliferation [1–5]. LIF also exerts its effects on blastomeres, embryonic carcinoma cells, extraembryonic cells, hematopoietic tissues/cells, bone, hepatocytes, neuron progenitors, and lipid metabolism [6–21]. This variety of biological properties is reflected by a diverse nomenclature developed in multiple laboratories.

In mice, LIF increased the percentage of blastocysts reaching the hatched stage in vitro when starting with eight-cell embryos cultured in 1000 U/ml of human recombinant LIF for 5 days [22]. Further studies demonstrated that both murine and human recombinant LIF significantly stimulated blastocyst formation and decreased embryo fragmentation/degeneration when added simultaneously at the initiation of in vitro culture of two-cell mouse embryos [23, 24]. LIF effects showed both dose and time dependency [23].

In addition to the positive embryotrophic effects demonstrated under in vitro coculture conditions, LIF appeared to be essential for murine implantation. In elegant knockout studies, females lacking a functional LIF gene were fertile, but their blastocysts failed to implant. Those blastocysts, however, were viable and implanted and developed to term when transferred to wild-type pseudopregnant recipients [6].

In the mouse, uterine expression of LIF by the endometrium peaks at the time of ovulation and just before implantation on the fourth day of pregnancy [25]. LIF is produced in the endometrial glands and might act as a paracrine factor within the endometrium. LIF targets are believed to be the endometrium and the embryo. LIF secretion appears to be required for the endometrium to undergo decidualization and for implantation of the blastocyst [26]. In humans, the expression of the LIF receptor beta is restricted to the luminal epithelium [27].

LIF transcripts have also been detected in the preimplantation stage blastocyst in the mouse (3.5 days postcoital), and LIF receptors have been found on the 4-day-old mouse embryo [28]. LIF affects the production of certain proteases implicated in the process of embryonic trophoblast invasion of the endometrial tissues during implantation. Both urokinase plasminogen activators and matrix metalloproteinase-9 levels increase under the influence of LIF in the mouse embryo on Day 3 of culture [29]. Recently, LIF and LIF receptor transcripts were detectable in human preimplantation embryos (with maximal expression on Day 2 embryos and Day 8 embryos and at the morula stage) [30]. Conflicting effects of LIF on human cytotrophoblasts have been reported, including diversion to the anchoring phenotype rather than the invasive pathway [31]. Therefore, LIF may be able to affect implantation and embryo development in an autocrine or a paracrine manner through endometrial (principally) and embryonic effects.

In the present murine studies, we aimed to assess the effect on in vivo implantation and embryo-fetal development of LIF and an anti-LIF polyclonal antibody (pab) when added at the time of blastocyst transfer to recipient females. Two-cell embryos were collected from B_6CBAF_1/J donors, cultured to the expanded blastocyst stage, and transcervically transferred to CD_1 (ICR) recipients. Specifically, we examined the effect of LIF and the pab on 1)

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TABLE 1. Effects of LIF and various doses of an anti-LIF pab on murine implantation, resorption, pregnancy, and viability rates.

Treatment	Implantation	Resorption	Pregnancy per transfer	Viability
LIF (5000 U/ml)	70/405 (17%)[a]	27/70 (38%)	15/27 (55%)[a]	43/405 (11%)[a]
Control	36/405 (9%)	21/36 (58%)	7/27 (26%)	15/405 (4%)
pab				
5000 U/ml	11/390 (3%)[a]	4/11 (36%)	2/26 (8%)	7/390 (2%)
2500 U/ml	22/225 (10%)	3/22 (14%)[a]	10/15 (66%)	19/225 (8%)[a]
1000 U/ml	20/210 (10%)	3/23 (13%)[a]	7/14 (50%)	17/210 (8%)[a]

[a] Significantly different from control.

implantation, resorption, pregnancy, and viability rates and 2) the overall structural and skeletal developmental of viable fetuses. LIF and/or its antibody could be used to enhance or inhibit the number of embryos capable of achieving successful implantation and normal development following uterine transfer.

MATERIALS AND METHODS

Embryos from 6- to 8-wk-old mice (*Mus musculus*) were used for this project. The CD_1 (ICR) (Charles River Laboratories, Wilmington, MA) and B_6CBAF_1/J (Jackson Laboratory, Bar Harbor, ME) mice were given water and food ad libitum. Females from both strains were mated with CD_1 (ICR) males (at least 10 wk of age). All mice were maintained in an animal facility at 25°C on a 14L:10D cycle.

A modified Krebs bicarbonate-buffered medium (mKBB) supplemented with 0.4% (4 mg/ml) BSA (Sigma, St. Louis, MO) was used for collection, culture, and transfer of embryos, as described previously [32]. The culture medium was filter sterilized through a 0.22-μm cellulose acetate membrane filter (Corning Inc., Corning, NY), dispensed into 25-cm^2 tissue culture flasks (Corning), and refrigerated at 0–4°C until utilized. The culture medium was equilibrated by incubating at 37°C in 5% CO_2 in 100% humidified air for at least 2 h prior to use.

Superovulation of female donors was induced by i.p. injection of 5 IU eCG (Sigma) followed in 48 h by 5 IU hCG (Sigma). At the time of hCG injection, each donor was individually caged with a proven fertile CD_1 male. The following morning, approximately 16 h after hCG injection, the female mice were inspected for vaginal copulatory plugs (Day 0 of pregnancy). On the morning of Day 1, plug-positive females were killed by cervical dislocation and the abdominal skin was retracted. Under sterile conditions, the abdominal wall was opened and the oviducts were removed and placed in 2 ml mKBB culture medium in sterile 35- × 10-mm polystyrene dishes. Using 20–5× magnification on a dissecting microscope (Zeiss Urban Quadrascope; Carl Zeiss, Thornwood, NY), two-cell embryos were removed from the oviducts by irrigating the fimbriated end of the oviduct with a 30-gauge needle connected to a 1-ml tuberculin syringe filled with mKBB medium until the embryos were expelled. Morphologically normal two-cell embryos were collected and cultured in mKBB medium for 72 h at 37°C in 5% CO_2 in 100% humidified air.

The injection schedule for female recipients was 24 h later than that for donors. Recipients were placed with vasectomized CD_1 males on the afternoon of hCG injection and examined for copulatory plugs the following morning to determine pseudopregnancy. On Day 4 of embryo development (Day 3 of pseudopregnancy), the pseudopregnant recipient females (surrogate uteri) were tranquilized using 0.1 mg/kg body mass acepromazine maleate (10 mg/ml Promace; Henry Schein, Melville, NY). For embryo transfer, Clay Adams PE10 tubing (Baxter Scientific, Columbia, MD) was square cut at the end (no beveling) and connected to a 30-gauge needle fitted to a 500-μl threaded Hamilton syringe (1750TP; Hamilton, Reno, NV). The PE10 tubing and syringe were filled with mKBB medium devoid of air bubbles. Approximately 1 cm of air was pulled into the distal tip of the tubing followed by 3 cm (20 μl) of medium containing 15 expanded blastocysts from the donor group. A final 0.5 cm of air was drawn into the transfer tube. Embryos were collected in as little medium as possible to reduce the risk of having them wash back out of the uterus upon removal of the transfer tubing. The cervical os was visualized using a glass speculum constructed from a Pasteur pipette. Using a Wild-Heerbrugg dissecting microscope (Sciscope Co., Iowa City, IA), the distal end of the embryo transfer tube was inserted approximately 2 cm into the cervical os, and the embryos were forced out of the tube by increasing the pressure in the Hamilton syringe until the proximal air spacer was observed passing the cervical os. The transfer tubing was gently removed, and females were caged individually and allowed to recover from tranquilization. The transfer tubing was rechecked under the microscope to ensure delivery of all embryos to the uterus.

Murine recombinant LIF (rmLIF) and a goat anti-murine recombinant LIF pab were purchased from R&D Systems (Minneapolis, MN). Test treatments were 1) 5000 U/ml rmLIF, 2) 5000 U/ml pab, 3) 2500 U/ml pab, and 4) 1000 U/ml pab. The lowest concentration of rmLIF (1000 U/ml) was previously determined to enhance the development of preimplantation mouse embryos. We selected this concentration as the lowest and examined the two higher concentrations to investigate potential effects. Multiple replicates for each of the test groups and controls were generated for each of multiple stimulation cycles. The neutralization $dose_{50}$ (ND_{50}) for the pab (activity) was defined as the concentration necessary to produce one-half maximum inhibition of rmLIF activity in a responsive cell line. The cell line utilized by R&D Systems for this purpose was a murine myeloid leukemia line known as DA-1a. Murine LIF was shown to enhance proliferation of these cells with a typical ED_{50} of 0.03–0.1 ng/ml. The ND_{50} for the pab was 0.1–0.2 μg/ml in the presence of 0.5 ng/ml rmLIF (approximately 1 U; R&D Systems). LIF and the pab solutions were prepared in mKBB to achieve the desired concentrations in the 20-μl volume transferred to the uterus with the blastocysts. Control females were established by a sham procedure in which 15 embryos were transferred in 20 μl of mKBB medium transcervically as previously described.

On Day 17 of pregnancy, females were killed by lethal overdose of i.p. sodium pentobarbital (64.8 mg/ml; Anpro Pharmaceuticals, Henry Schein). The abdominal skin of the females was removed, and the abdomen was opened to externalize the uterus. The uterus was inspected for number of implantation sites (viable = fetuses; nonviable = resorption sites) prior to removal. Amniotic membranes were stripped, and fetuses were separated from each placenta and analyzed.

The following data were recorded: number of fetuses, fetal tail length, fetal crown-rump length, fetal mass, placental mass, placental linear dimensions, and number of resorption sites. Length was measured in millimeters, and mass was measured in grams. The following definitions were used: 1) implantation rate: number of implantation sites (both viable pups and resorbed fetuses)/total number of embryos transferred in the group; 2) pregnancy rate: number of pregnant females (i.e., the number of females with pups that appeared viable or morphologically normal at Day 17)/total number of females transferred in the group; 3) resorption rate: number of amorphous resorption sites or fetuses demonstrating severe macroscopic developmental retardation/the total number of implantation sites (both viable and resorbed); and 4) viability rate: number of viable fetuses/total number of embryos transferred for the group.

Fetuses were removed from amniotic membranes and separated from each placenta, and various measurements were taken. Cartilage and bone were differentially stained to reveal any gross skeletal defects. Once removed from the uterus, the fetuses were fixed in 10% neutral buffered formalin for at least 24 h. After fixation, evisceration was accomplished using watchmaker forceps. At this time, the fetuses were washed in several changes of distilled water to remove most of the formaldehyde. Following washing, the fetuses were placed in an alcian blue (Sigma) solution (10 mg alcian blue, 70 ml 100% ethanol, 30 ml glacial acetic acid) for 12–48 h [33]. Once the cartilage was stained, the dehydration step was accomplished by placing the fetuses in a minimum of two absolute alcohol baths for at least 24 h. Fetuses were rehydrated by a 2-h exposure to a series of baths consisting of decreasing concentrations of ethanol (i.e., 75%, 50%, and 25%) followed by two consecutive distilled water baths for 1 h each. After rehydration, fetuses were partially macerated for 1–2 h in a solution containing 1 g of trypsin (Fisher Scientific, Norcross, GA), 30 ml saturated aqueous sodium borate (Fisher), and 70 ml distilled water. The fetuses were left in this solution until the soft tissues became transparent and the fetuses had a consistency of warm gelatin. The bone tissue was stained by placing fetuses in alizarin red (Sigma) solution (0.5% KOH, 0.1% alizarin red in water) for 24 h. Thereafter, the fetuses were placed in a 25%

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TABLE 2. Effects of LIF and various doses of an anti-LIF pab on murine feloplacental development.

Treatment	Body mass (g)	Crown-rump length (mm)	Tail length (mm)	Placental length (mm)	Placental width (mm)	Placental mass (g)
LIF (5000 U/ml)	0.53 ± 0.1	17.6 ± 2.1	6.9 ± 1.3	9.5 ± 1.3	9.3 ± 1.1	0.16 ± 0.05
Control	0.58 ± 0.1	17.7 ± 2.9	7.0 ± 1.4	9.8 ± 1.1	9.5 ± 0.9	0.17 ± 0.04
pab						
5000 U/ml	0.45 ± 0.07[a]	16.6 ± 0.7	6.9 ± 0.3	9.7 ± 1.6	9.0 ± 1.0	0.2 ± 0.08
2500 U/ml	0.73 ± 0.2	18.2 ± 2.1	7.7 ± 1.3	8.8 ± 0.7[b]	9.8 ± 1.6	0.17 ± 0.05
1000 U/ml	0.63 ± 0.2	17.3 ± 2.1	7.4 ± 2.0	8.5 ± 0.9[a]	9.0 ± 1.5	0.15 ± 0.03

[a] Significantly different from pab 2500 U/ml.
[b] Significantly different from control.

glycerin in 0.5% aqueous KOH solution for 24 h. To this solution, three to five drops of 3% hydrogen peroxide per 100 ml glycerin were added.

Fetuses were then placed in succeeding solutions of increasing glycerin concentrations (50% glycerin with 0.5% KOH, 75% glycerin with 0.5% KOH, and 100% glycerin), each for 24 h. Specimens were stored in glycerin to which a few crystals of phenol or thymol were added [33]. Fetuses were then examined and photographed using a Wild-Heerbrugg dissecting microscope. Humerus length, length of diaphyseal ossification in the humerus, width of dorsal gap between the vertebral pedicles or the vertebral space, estimated percentage of ossification in the exoccipital bone, length of ipsilateral scapula measured from vertebral border to glenoid cavity, and length of ossification center in scapular spine were recorded.

The results of treatments (LIF, pab, and control conditions) on implantation, resorption, viability, and pregnancy rates were compared using 2 × 4 contingency tables (two-tailed) and chi-square analysis as appropriate. ANOVA (followed by the Bonferroni *t*-test for multiple comparisons) was utilized to assess differences in fetoplacental and skeletal measurements in response to the various treatments. Results are presented as mean ± SD. Differences were considered significant when P values were ≤0.05 .

RESULTS

Table 1 presents the results of LIF and the anti-LIF pab treatments on implantation, resorption, pregnancy, and viability rates. Treatment with LIF (5000 U/ml) resulted in significantly increased implantation (17% versus 9% in controls, $P = 0.0005$), pregnancy (55% versus 26% in controls, $P = 0.05$), and viability (11% versus 4% in controls, $P = 0.0002$) rates when compared with the nontreated conditions.

Exposure to a similar dose of the pab (5000 U/ml) produced a significant reduction in the implantation rate (3% versus 9% in controls, $P = 0.0005$) and a decrease in the pregnancy rate that reached borderline significance (8% versus 28%, $P = 0.07$). However, the lower pab doses resulted in a significant reduction in the resorption rate (14% and 13%, respectively, versus 58% in controls, $P = 0.0008$ for both) and an increase in the viability rate (8% for both versus 4% in controls, $P = 0.01$).

The number of viable pups per individual female varied for each group. Controls ranged from zero to four fetuses per female with an average of 0.56 fetuses/female. For the LIF treatment group, the range was zero to six fetuses per female (average of 1.6 fetuses/female). The three pab test groups demonstrated the following individual variability: 1) 1000 U/ml group: zero to five fetuses per female (average of 1.2 fetuses/female); 2) 2500 U/ml group: zero to four fetuses per female (average of 1.3 fetuses/female); 3) 5000 U/ml group: zero to four fetuses per female (average of 0.25 fetuses/female).

The effects of LIF and pab on fetoplacental development are shown in Table 2. LIF treatment had no significant impact on any of the fetal or placental parameters assessed. In contrast, the pab treatments had a significant overall negative effect on placental length ($P = 0.002$). Specifically, at 2500 and 1000 U/ml the pab significantly decreased placental length ($P < 0.05$ and $P < 0.01$, respectively, versus controls).

Table 3 presents the results of LIF and pab treatments on murine skeletal development. LIF had no impact on any of the bone measurements. Conversely, pab treatments had a significant overall effect on the ossification of the exoccipital bone and on vertebral space width ($P = 0.0001$ for both parameters). Although the effects of the various pab doses were heterogeneous, significant differences depicting a negative effect on defined areas of bone development were demonstrated. At 5000 U/ml, the pab resulted in a significant reduction in the ossification of the exoccipital bone as compared with controls ($P < 0.01$). At 2500 U/ml and 1000 U/ml, the pab produced significant variation in vertebral space width ($P < 0.01$ for both doses).

DISCUSSION

LIF plays a critical role in murine reproduction by enhancing in vitro preimplantation embryo development [22, 23] and is an absolute requirement for implantation [6, 25]. LIF stimulates mouse embryo development in vitro in a dose- and time-dependent manner. At a dose of 1000–100 000 U/ml for two-cell embryos, LIF produced maximal stimulation of blastocyst formation and decreased the percentage of degenerating embryos. These effects were also mouse strain dependent [23]. Both LIF and its receptors

TABLE 3. Effects of LIF and various doses of an anti-LIF pab on murine skeletal development.

Treatment	Humerus length (mm)	Humerus diaphyseal ossification center (mm)	Vertebral space width (mm)	Ossification exoccipital bone (%)	Ipsilateral scapula length (mm)	Ossification center of scapular spine length (mm)
LIF (5000 U/ml)	3.0 ± 0.2	0.9 ± 0.2	0.8 ± 0.2	82.7 ± 29	2.4 ± 0.3	0.9 ± 0.2
Control	3.0 ± 0.5	1.0 ± 0.5	0.8 ± 0.2	88.6 ± 24	2.4 ± 0.4	1.0 ± 0.4
pab						
5000 U/ml	2.8 ± 0.3	0.6 ± 0.1	0.9 ± 0.2	47.0 ± 31[a]	2.3 ± 0.2	0.6 ± 0.1
2500 U/ml	3.4 ± 0.5	1.3 ± 0.4[b]	1.0 ± 0.1[a]	95.0 ± 13	2.8 ± 0.5[b]	1.3 ± 0.4[b]
1000 U/ml	3.3 ± 0.5	1.3 ± 0.5[b]	1.0 ± 0.1[a]	90.0 ± 26	2.7 ± 0.6[b]	1.2 ± 0.5[b]

[a] Significantly different from control.
[b] Significantly different from pab 5000 U/ml.

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seem to be operative in the embryo (at early stages of development) and the endometrium (at the time of implantation), making possible auto-paracrine cross-talk pathways. The addition of LIF to blastocysts at the time of uterine transfer could enhance in vivo embryo development/implantation.

In the present study, LIF (at 5000 U/ml) significantly stimulated blastocyst implantation, leading to enhanced pregnancy (per transfer) and viability (per embryo) rates. This is the first report of such an effect using in vivo-generated murine embryos grown to blastocysts under in vitro culture conditions and transcervically transferred to the uteri of synchronized female recipients.

Surgical transfer of embryos to the uterine horns [34, 35] or to the fimbriated end of the oviduct [36, 37] are common techniques, requiring anesthesia and laparotomy, and as many as 70–80% of these embryos develop to term [38]. We developed a novel and successful nonsurgical method of murine embryo transfer, transcervical blastocyst transfer. This procedure is a modification of a technique described previously for which development to term was approximately 30% [39]. The efficiency of our technique is demonstrated by the adequate pregnancy rate achieved in controls (26%).

The present results also extend the observations of Fry et al. [40], who studied the effect of culturing ovine embryos at the morula stage in 1000 U/ml LIF. LIF treatment resulted in an increase in the number of hatching blastocysts and a higher pregnancy rate when treated embryos were transferred to recipient females. The stimulatory activity of LIF may be due to embryonic/trophoblastic or endometrial effects because LIF receptors are present in both target tissues.

These initial results demonstrate that LIF enhances murine implantation rates following blastocyst transfer to the uterus. We are studying whether this effect of LIF is also present following the transfer of in vitro-generated embryos. The positive impact of LIF on the number of implanted embryos that developed to viable fetuses was not accompanied by any deleterious effects on overall fetal skeletal development or placental growth. Although the precise role(s) of LIF in humans has not been established, it is tempting to speculate that LIF could be used to optimize human blastocyst transfer at the time of in vitro fertilization therapy. However, although LIF levels are relatively constant in the human fallopian tube, elevated levels have been associated with ectopic pregnancies [41].

At a concentration of 5000 U/ml, the anti-LIF pab produced a significant inhibition of the implantation rate when compared with control conditions. This inhibition is probably the result of a blockage of ''native'' LIF (endometrial or embryonic) effects. Paradoxically, the lower concentrations of the pab (2500 and 1000 U/ml) resulted in a significant decrease in the resorption rate and a consequent increase in the viability rate. Further investigation is required to explain these observations. Nonspecific effects cannot be ruled out. However, R&D Systems, using a direct ELISA, found that this particular pab demonstrated no cross-reactivity when tested with more than eight other recombinant murine (rm) cytokines, including rmIL-1α and β, rmGM-CSF, and rmTNFα and β, as well as over 50 recombinant cytokines from other animal models (R&D Systems, personal communication). Based on our current data, we cannot explain the apparently paradoxical effect observed with pab [42].

At the high dose studied, the anti-LIF pab demonstrated potential contraceptive activities by possibly preventing murine embryo implantation. However, there was a general deleterious effect of the pab on placental growth (decrease of placental width) and skeletal development (decrease of the ossification of the exoccipital bone and increase in vertebral space width) of the viable fetuses. LIF increases the resorption of bone [43–45], and high levels have been associated with pathologic conditions such as cachexia, tissue calcification, pancreatitis, and gonadal and thymic anomalies [11]. LIF also inhibited bone nodule formation in fetal rat calvaria cell cultures [46] and inhibited growth and mineralization of early fetal mouse long bone cultures [47]. In our study, LIF did not seem to affect bone development. Nevertheless, the anti-LIF pab showed deleterious effects that were heterogeneous and not dose dependent.

The addition of LIF at the time of transcervical blastocyst transfer resulted in a significant enhancement in implantation and pregnancy rates. Both LIF and anti-LIF pab have pronounced effects on implantation and fetal development in mice.

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