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Leukemia inhibitory factor is expressed by the preimplantation uterus and selectively blocks primitive ectoderm formation *in vitro*

(differentiation/embryonic stem cell/embryoid body/cytokine)

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ABSTRACT **Among its many activities, leukemia inhibitory factor (LIF) can maintain embryonic stem cell monolayers in a pluripotent undifferentiated state. Presuming that this might reflect its physiologic role during embryogenesis, we have examined LIF expression in the embryonic environment by RNase protection assays and have determined its *in vitro* effect on differentiating embryonic stem cell embryoid bodies. Of all adult tissues analyzed, LIF transcripts appear only in the uterus, where their level fluctuates with the estrous cycle, peaking after ovulation. LIF expression continues in the uteri of pregnant and pseudopregnant females, with a relative peak when blastocysts are normally present. As for its effects on *in vitro* differentiation, we have found that LIF blocks embryoid body differentiation only partially, yet in a precise manner. Using molecular markers to follow the differentiation of defined cell types, we demonstrate that LIF selectively inhibits the formation of primitive ectoderm, while permitting the differentiation of primitive endoderm. These results suggest a specific role for LIF in preimplantation mouse development.**

Previous studies have suggested that the cytokine leukemia inhibitory factor (LIF) may be involved in early mouse development, based on its ability to inhibit monolayer differentiation of embryonic stem (ES) cells (1, 2). In addition, reverse transcriptase/PCR assays have shown LIF expression by blastocysts (3, 4), and *in situ* and RNase protection data have demonstrated expression by post-implantation egg cylinders (3, 5) and by the pregnant uterus (6). Here, we further examine LIF expression in the embryonic environment and present evidence for increased LIF expression by the uterus shortly after ovulation and just before blastocyst implantation.

Since LIF is expressed by the uterus during the preimplantation period, it may influence embryonic development. To investigate how LIF might function in the early embryo, we have used the pluripotential differentiation of ES cells, which resemble inner cell mass (ICM) cells (7), as an *in vitro* system for the generation of early embryonic cell types. Although ES cells in monolayer culture possess a limited differentiative capacity in the absence of LIF (8), aggregates of ES cells in suspension culture form embryoid bodies that spontaneously differentiate into a wide range of embryonic cell types (9). These cell types include the extraembryonic visceral and parietal endoderm, which arise from primitive endoderm, and mesodermal cell types such as cardiac muscle and blood, which are primitive ectoderm derivatives. We have followed the expression of molecular markers for these distinct cell types to compare embryoid bodies differentiated in the absence or presence of a standard concentration of LIF. Even though LIF prevents differentiation of ES cells in monolayer culture (1, 2), we find that LIF does not block all differentiation pathways in ES embryoid bodies but instead exerts a selective inhibitory effect.

MATERIALS AND METHODS

Tissue Isolation. We staged virgin Swiss–Webster female mice (Taconic Farms) in the estrous cycle by using vaginal smears that were stained using the Diff-Quik stain set (Baxter Scientific Products, McGaw Park, IL) and scored for the presence of leukocytes, nucleated epithelial cells, cornified epithelial cells, and mucus (10). Individual uterine horns were dissected away from the ovaries and oviducts. Uteri were similarly dissected from preimplantation pregnant and pseudopregnant mice, which were obtained by mating with vasectomized males. At day 3.5 post coitum (p.c.), blastocysts were recovered to verify the developmental staging, where day 0.5 p.c. is defined as noon of the day of the vaginal plug. For post-implantation pregnant mice, uterine material was dissected free from the decidua, and the egg cylinders were staged.

RNase Protection Analysis. Total RNA was isolated by standard methods (11). SP6, T3, and T7 antisense probes were synthesized and hybridized to RNA samples as described (12). For comparable signals, we synthesized ribosomal protein L32 probes at 10% the specific activity of other probes. Expression levels were analyzed by densitometric scanning (Molecular Dynamics model 300A computing densitometer with IMAGEQUANT 2.0 software). Probes for α-fetoprotein (13), *H19* (14), collagen type IV (15), laminin B1 (15), *Oct-3* (16), ζ-globin (17), and α-cardiac actin (18) have been described. The probe for *Fgf-5* was derived by linearization of the cDNA clone (19) with *Bst*NI, resulting in a probe that protects 151 nucleotides (nt). The probe for *REX-1* was obtained by linearization of pREX-gem (20) with *Xho* I, resulting in a probe that protects 117 nt. The ribosomal protein L32 probe was derived from the processed pseudogene 4A (21), using the *Xho* II–*Dra* I fragment subcloned into Bluescript IIKS(+) (Stratagene) and linearized at the *Pvu* II site, resulting in a probe that protects 83 nt. To detect total LIF transcripts, we used an antisense probe synthesized from a 280-base-pair (bp) exon fragment [bp 3429–3708 of the genomic sequence (22)] cloned by PCR amplification from BALB/c genomic DNA into pGEM-7zf (Promega). To distinguish between the two LIF transcripts that use alternative first exons (23), we started with a PCR-amplified cDNA–genomic hybrid clone in which the "diffusible" exon 1 is fused to exon 2, but which contains the intron between exons 2 and 3. From this hybrid, we subcloned into Bluescript IIKS(+) a 286-bp fragment that starts at bp 954 of the genomic sequence 5′ of exon 1 and extends to the *Eco*47III site at bp 2798 in the intron between exons 2 and 3 (22). This probe protects 209 nt for transcripts encoding the diffusible

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Abbreviations: LIF, leukemia inhibitory factor; ES, embryonic stem; ICM, inner cell mass; p.c., post coitum; nt, nucleotide(s).

form of LIF and 181 nt for transcripts encoding the extracellular matrix-associated form.

Embryoid Body Differentiation. We maintained early-passage ES cells on mitomycin C-treated feeder layers of STO embryonic fibroblasts in standard ES culture medium (24). Prior to embryoid body formation, ES cells were passaged at least once on gelatinized tissue culture dishes in ES medium supplemented with purified recombinant mouse LIF (GIBCO/BRL) at 1000 units/ml, to eliminate STO cells from the cultures. These ES monolayers were also harvested for RNA preparation and analysis. We compared embryoid body differentiation in the absence and presence of LIF in seven experiments, using either the D3 (9) or CCE (25) cell lines. To initiate embryoid body aggregation, we plated 1×10^6 trypsinized ES cells in single-cell suspension in bacteriological dishes (Fisher), using ES culture medium without or with LIF (GIBCO/BRL) at 1000 units/ml. The embryoid bodies were fed by replacement of 50% of the medium every other day; LIF-treated embryoid bodies were maintained in LIF (1000 units/ml) throughout the experiment.

Immunohistochemistry. Embryoid bodies were washed three times in phosphate-buffered saline and frozen in OCT embedding medium (Miles). Sections (12 μm) were fixed in acetone and stained using a standard immunoperoxidase protocol (26). The TROMA-1 (27) and SSEA-1 (28) monoclonal antibodies were obtained as ammonium sulfate-precipitated hybridoma supernatants (Developmental Studies Hybridoma Bank, Baltimore) and used at a 1:100 dilution. Biotinylated goat secondary antibodies (Jackson ImmunoResearch) were used at a 1:10,000 dilution.

RESULTS

LIF Expression by the Preimplantation Uterus. To investigate LIF expression, we used a RNase protection assay to examine RNA from dissected adult and embryonic mouse tissues. In these experiments, we employed three probes that were included in each hybridization. We used two nonoverlapping probes for LIF, one that detects total transcripts and the other that protects fragments of different sizes corresponding to the two transcripts that encode diffusible and extracellular matrix-associated forms of LIF (23). The third probe detects transcripts for ribosomal protein L32 (21), used as an internal standard. Using these probes, we were unable to detect significant levels of LIF expression in most adult tissues examined, including bone marrow, brain, heart, kidney, skeletal muscle, liver, lung, salivary gland, seminal vesicle, small intestine, spleen, stomach, testis, and thymus (data not shown). We also examined post-implantation embryonic tissues and found that LIF expression occurs in extraembryonic tissues of trophectodermal origin but not at detectable levels in the fetus (unpublished data), consistent with previous results (3, 5).

In contrast, we did observe LIF expression in the uterus of adult virgin females (Fig. 1), which were staged in the estrous cycle by vaginal smears (10). Based on histological criteria, the mouse estrous cycle can be divided into five stages, where diestrous represents the quiescent phase, proestrous and estrous represent the proliferative phases of the endometrium, and metestrous 1 and metestrous 2 represent the degenerative phases, with ovulation occurring during estrous. LIF expression appears low during diestrous, proestrous, and metestrous 2, but we found that levels are significantly elevated (>6-fold) in animals during estrous and metestrous 1 (Fig. 1). At these stages, the transcript encoding diffusible LIF is expressed at higher levels than that encoding matrix-associated LIF.

We also examined whether uterine LIF expression varies during pregnancy (Fig. 2). At day 0.5 p.c., uterine expression of LIF was found at levels similar to those in virgin females

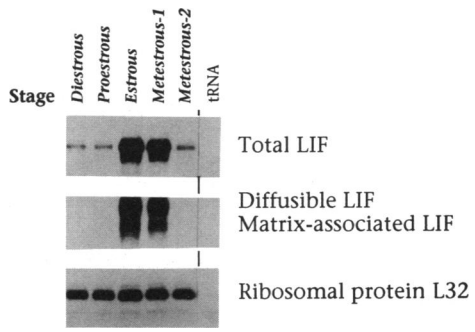

FIG. 1. Estrous cycle variation of uterine LIF expression, analyzed by RNase protection. Each hybridization contained two antisense probes for LIF and an antisense probe for ribosomal protein L32 as an internal standard. Total RNA (20 μg) was used, with an autoradiographic exposure of 7 days for LIF and 1 day for ribosomal protein L32. We examined a total of 27 mouse uteri, of which 5 representatives of the indicated stages are shown. In one case, we observed intermediate levels of LIF expression in a mouse that was staged as early metestrous 2 (data not shown).

at estrous and metestrous 1, correlating with female receptivity for mating from proestrous to metestrous 1 (10). At days 1.5 and 2.5 p.c., when the embryo is in the oviduct, LIF expression declines, followed by higher levels of expression at day 3.5 p.c., when blastocysts are found in the uterus. Again, the transcript encoding diffusible factor appears more abundant. After implantation at day 4.5 p.c., however, LIF expression becomes nearly undetectable. Interestingly, this pattern of LIF expression is not dependent on the presence of embryos, as it is paralleled in pseudopregnant females (Fig. 2).

LIF Permits Differentiation of Primitive Endoderm Derivatives *in Vitro*. We have also explored the potential function of LIF by comparing in parallel the differentiation of ES embryoid bodies in the absence or continual presence of purified recombinant LIF at 1000 units/ml, a concentration normally used to maintain undifferentiated ES monolayers (2). When plated in bacteriological dishes, D3 ES cells (9) aggregated to form embryoid bodies with equal efficiencies in the absence or presence of LIF in standard ES culture medium (data not shown). After a few days of suspension culture, both control and LIF-treated embryoid bodies started to form outer endodermal layers. After 7–10 days, however, many control embryoid bodies cavitated and produced large cystic structures resembling visceral yolk sacs, whereas LIF-treated bodies cavitated much less frequently and rarely formed cystic structures (data not shown).

We confirmed these morphological differences by analyzing the expression of cell-type-specific marker genes during suspension culture (Fig. 3). These marker genes were selected on the basis of published *in situ* hybridization experiments demonstrating the desired specificity *in vivo*. Thus, we used α-fetoprotein to mark visceral endoderm, which specifically expresses this gene in early post-implantation embryos (13, 29). The appearance of high levels of α-fetoprotein suggests that visceral endoderm forms efficiently in both control and LIF-treated embryoid bodies (Fig. 3). We obtained similar results using *H19*, which is expressed by both visceral and parietal endoderm in early post-implantation embryos (day 6.5 p.c.) but not by primitive ectoderm or newly formed mesoderm (14, 30). Parietal endoderm also forms in both control and LIF-treated embryoid bodies, as indicated by increased expression of collagen type IV and laminin B1 (31). These results indicate that primitive endoderm derivatives can form in the presence of LIF at 1000 units/ml.

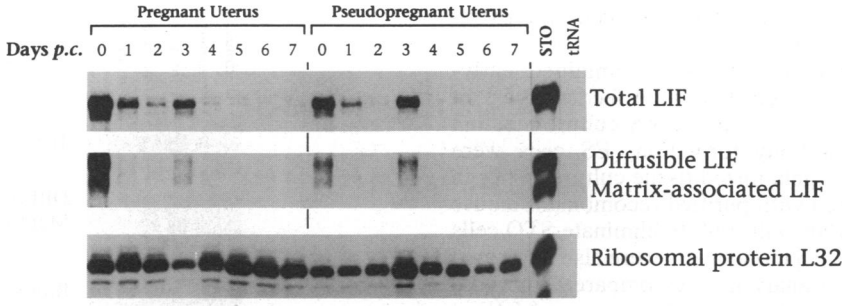

FIG. 2. Uterine LIF expression in individual pregnant and pseudopregnant mice. Probes and exposure times are the same as in Fig. 1. The indicated days on the figure are a half day less than the actual number of days p.c.; thus, day 3 as labeled on the figure represents 3.5 days p.c. We analyzed 54 mouse uteri, of which 16 representative examples are shown. Each time point has been examined from at least two mice; in particular, 5 uteri were analyzed from pregnant mice at day 3.5 p.c. RNA from the STO embryonic fibroblast cell line is included for comparison, as STO cells produce sufficient LIF to inhibit ES monolayer differentiation (1, 2) and express roughly equivalent levels of transcripts for diffusible and matrix-associated LIF (23).

In contrast, LIF treatment completely blocked expression of two early markers of mesodermal differentiation (Fig. 3). One marker is ζ-globin (17), which is expressed *in vivo* by primitive erythrocytes in blood islands at day 7.5 p.c. (A. Leder, A. Kuo, M.M.S., unpublished data). A second marker is α-cardiac actin, which is expressed by newly formed cardiac and skeletal striated muscle in embryos at day 9.0 p.c. (18). Although both markers are expressed early in the differentiation of control embryoid bodies, they are never expressed at significant levels by LIF-treated bodies (Fig. 3), even after 3 weeks of culture or after reattachment to tissue culture surfaces (data not shown). It therefore appears that LIF can block the differentiation of mesodermal derivatives. The examination of other genes expressed by early mesoderm derivatives, including *GATA-1* and *T* (brachyury), also yields results consistent with this conclusion (unpublished data).

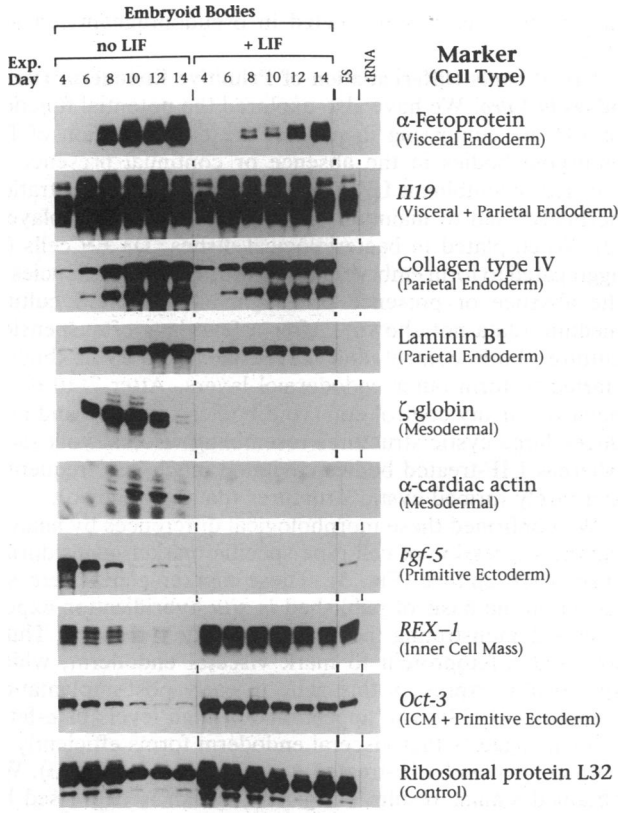

FIG. 3. Expression of lineage-specific marker genes in differentiating ES embryoid bodies. For each RNase protection shown, we used the following amounts of total RNA and autoradiographic exposure times: α-fetoprotein and *H19*, 15 μg and 22 h; laminin B1 and collagen type IV, 15 μg and 4 h; ζ-globin, 20 μg and 30 h; α-cardiac actin, 20 μg and 4 days; *Fgf-5*, 20 μg and 29 h; *REX-1*, 20 μg and 4 h; *Oct-3*, 15 μg and 4 h; ribosomal protein L32, 15 μg and 20 h.

LIF Blocks Primitive Ectoderm Formation *in Vitro*. To determine more precisely the point at which LIF exerts its inhibitory effect, we investigated the expression of *Fgf-5*, which is expressed in primitive ectoderm and cells of the primitive streak before and during gastrulation *in vivo* (19, 32). We observed that *Fgf-5* expression increases transiently in control embryoid bodies but is not expressed in LIF-treated bodies (Figs. 3 and 4), suggesting that primitive ectoderm cells do not arise. We also examined expression of *REX-1*, which is expressed by undifferentiated ES cells and by the blastocyst ICM but not by primitive endoderm, primitive ectoderm, or their derivatives (20). Expression of *REX-1* decreases during differentiation of control embryoid bodies but remains high in LIF-treated bodies (Figs. 3 and 4). These observations also correlate with the expression pattern of *Oct-3* (Figs. 3 and 4), a gene expressed in both ICM and primitive ectoderm *in vivo* (16, 33). These results support the hypothesis that LIF selectively blocks ES cell differentiation into primitive ectoderm.

Finally, we also examined LIF expression during embryoid body differentiation, since it was previously reported that ES monolayers deprived of LIF displayed a strong transient

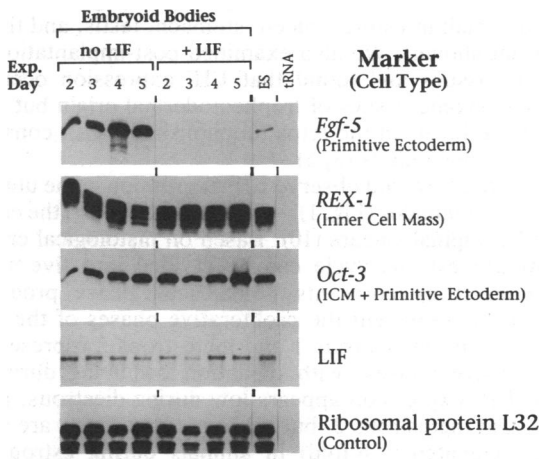

FIG. 4. Expression of early lineage-specific marker genes in differentiating ES embryoid bodies. We used the following amounts of total RNA and autoradiographic exposure times: *Fgf-5*, 20 μg and 29 h; *REX-1*, 20 μg and 4 h; *Oct-3*, 15 μg and 4 h; LIF, 20 μg and 5 days; ribosomal protein L32, 20 μg and 30 h. The LIF probe used protects total LIF transcripts.

increase in LIF expression, peaking 3 days after LIF removal (5). In contrast, we found that ES embryoid bodies at days 2–5 of culture display LIF expression levels identical to those of undifferentiated ES monolayers, even though by day 5 the control embryoid bodies exhibit markers of primitive ectoderm and mesoderm differentiation (Fig. 4; unpublished data).

Immunohistochemical Analysis of Embryoid Bodies. We have also investigated the localization of cell types in individual embryoid bodies by immunohistochemical staining of frozen sections. To mark primitive endoderm and its derivatives, we used the TROMA-1 monoclonal antibody (27). As expected, TROMA-1 stains the outer endodermal layer of embryoid bodies in a pattern that is indistinguishable for control and LIF-treated embryoid bodies analyzed at day 6 (data not shown) and at day 10 (Fig. 5 *A* and *B*). This staining pattern confirms that primitive endoderm derivatives form normally in the presence of LIF. We also examined staining by the SSEA-1 monoclonal antibody, a marker for ICM and primitive ectoderm (28). At day 6 of culture, both control and LIF-treated embryoid bodies possess widespread SSEA-1 staining in their interiors (data not shown). By day 10, however, the control embryoid bodies show only limited staining (Fig. 5*C*), whereas the LIF-treated bodies retain extensive staining, even deep in their interior (Fig. 5*D*). These results indicate that the LIF-inhibited cells remain undifferentiated.

DISCUSSION

The expression of LIF by the preimplantation uterus suggests that maternal LIF might regulate early embryonic development. Several possible roles might be envisioned for LIF function, all consistent with the observation that uterine LIF transcripts primarily encode the diffusible form (Figs. 1 and 2). Thus, for example, the peak of uterine LIF expression after ovulation might exert an effect on the egg in the oviduct. Alternatively, uterine LIF might prepare the endometrium for blastocyst implantation (6), while later trophoblastic and placental LIF expression might support maternal sustenance of the embryo. Such a role might be similar to the proposed functions of the cytokine colony-stimulating factor 1 and its receptor c-*fms* in regulating trophoblast proliferation and differentiation (34).

More interestingly, however, uterine LIF expression at day 3.5 p.c. may influence stem cell differentiation within the blastocyst, a possibility consistent with our *in vitro* differentiation experiments. Thus, although fertilized eggs can be cultured to the blastocyst stage *in vitro* in defined medium without LIF, embryo viability in such experiments can be greatly improved by coculture with uterine cells (35). In particular, LIF treatment of embryos developing *in vitro* has been reported to cause late blastocysts to form larger trophoblasts and better defined ICMs (36), suggesting that LIF can affect the ICM even when surrounded by the trophectoderm. Furthermore, because LIF can inhibit *in vitro* differentiation within embryoid bodies many cell layers thick (Fig. 5*D*), it is conceivable that uterine LIF could exert similar effects within blastocysts.

In addition, the estrous cyclicity of uterine expression suggests that LIF is produced by the endometrium, perhaps regulated by ovarian steroid hormones. The high levels of LIF expression during estrous and metestrous 1 correspond to the secretory phases of the endometrium, shortly after the peak period of 17β-estradiol and progesterone synthesis by the ovary (37). During pregnancy, uterine LIF expression correlates well with levels of 17β-estradiol, including a relative peak at day 3.5 p.c. that is required for blastocyst implantation (38). Similar time courses of LIF expression in pregnant and pseudopregnant uteri have been described by Bhatt *et al.* (6), who also demonstrated LIF expression by endometrial glands using *in situ* hybridization and found that it can be regulated by estrogen (note that their nomenclature for days p.c. differs from ours). In contrast, however, Bhatt *et al.* (6) found no expression of LIF by nonpregnant uteri, including one uterus at the estrous stage. Although we are unable to account for this discrepancy, the estrous cyclicity that we observe supports their conclusion that LIF expression is under hormonal control.

In the course of investigating potential functions of LIF during embryogenesis, we have also characterized the *in vitro* differentiation of ES embryoid bodies using molecular markers of cell types. Our results indicate that embryoid body differentiation *in vitro* displays characteristics expected for lineage relationships found *in vivo*. Thus, expression of the primitive ectoderm marker *Fgf-5* transiently increases to peak at day 4 of culture and decreases during further differentiation (Figs. 3 and 4). Mesodermal markers (ζ-globin and

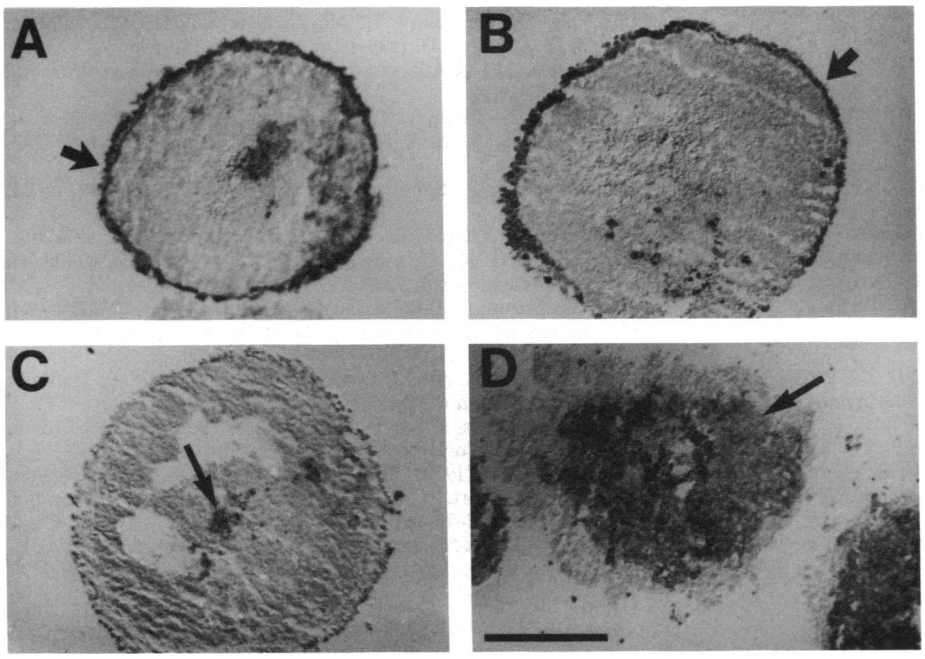

FIG. 5. Immunohistochemical staining of frozen sections of ES embryoid bodies by the TROMA-1 (27) and SSEA-1 (28) monoclonal antibodies. All panels depict typical staining patterns of embryoid bodies from day 10 of an experiment similar to that shown in Fig. 3. Darker areas represent positive immunoreactivity, and lighter regions are nonreactive embryoid body cells, counterstained with hematoxylin. The scale bar in *D* represents 0.2 mm. (*A*) Control embryoid body stained by TROMA-1. Most staining is evident at the rim of the embryoid body (arrow). (*B*) TROMA-1 staining of a LIF-treated embryoid body (arrow). (*C*) Embryoid body cultured in the absence of LIF, stained by the SSEA-1 antibody. Only limited staining in the embryoid body core is evident (arrow). (*D*) SSEA-1 staining of a LIF-treated embryoid body (arrow).

α-cardiac actin) appear at days 5–6 of culture and increase in level over the next few days (Fig. 3; unpublished data). Conversely, markers of ICM cells (*REX-1*) or ICM and primitive ectoderm (*Oct-3*) gradually decrease in expression. These time courses are consistent with the differentiation of cells from an initial ICM type through primitive ectoderm and then into mesoderm and other differentiated cell types.

In the presence of LIF, the formation of primitive ectoderm in embryoid bodies appears to be inhibited, although the differentiation of primitive endoderm derivatives is largely unaffected. Therefore, LIF can exert a selective effect on ES differentiation, allowing the formation of primitive endoderm from ICM-like cells but blocking their differentiation into primitive ectoderm. Although there seems to be a partial inhibition of visceral endoderm formation, as judged by α-fetoprotein expression (Fig. 3), we observed little or no inhibition of α-fetoprotein expression in experiments using a different cell line, CCE (25), suggesting that this effect may be specific for the D3 cell line (data not shown). Although higher concentrations of LIF might conceivably eliminate primitive endoderm formation in ES embryoid bodies, it is nonetheless clear that the formation of primitive endoderm and ectoderm *in vitro* is differentially affected by LIF. This differential response raises the possibility that it could be employed *in vivo* to regulate primitive ectoderm formation.

Such a role for LIF would differ from an earlier model, in which stem cell differentiation would be controlled by a negative feedback loop (5). These authors proposed that locally increased LIF activity produced by differentiated cells *in vivo* might inhibit further differentiation and, thereby, maintain the stem cell population. However, we have found no evidence for increased LIF expression during early ES embryoid body differentiation. Furthermore, the permissiveness of LIF for primitive endoderm differentiation suggests that feedback regulation, if it exists, is selective for primitive ectoderm formation. Our results indicate that LIF does not antagonize stem cell differentiation *per se* and, therefore, it is not a passive factor for the maintenance of stem cells. Instead, local concentrations of LIF in the uterus (6) and the blastocyst (3, 4) may be sufficient for it to influence ICM differentiation. If so, we can speculate that LIF might regulate the allocation of cells to the primitive endoderm versus the primitive ectoderm at the late blastocyst stage, either in a spatial or temporal fashion. The investigation of this possibility will require further characterization of the expression of LIF and similar genes and their receptors, as well as the elucidation of additional markers for early embryonic cell types.

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