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The Genes for Leukemia Inhibitory Factor and Interleukin-6 Are Expressed in Mouse Blastocysts prior to the Onset of Hemopoiesis

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We have investigated the role that hemopoietic regulatory molecules may play in mouse embryogenesis prior to the appearance of hemopoietic stem cells or their microenvironments. Using polymerase chain reaction analysis, we detected mRNA transcripts for interleukin-6 (IL-6) and leukemia inhibitory factor (LIF) but not for granulocyte-macrophage colony-stimulating factor (GM-CSF) or IL-3 in mouse blastocysts at 3.5 days of gestation. Functional IL-6 protein was also detected in cultured blastocysts as a secreted product, as was an activity consistent with the presence of LIF protein. The expression of IL-6 and LIF in blastocysts prior to hemopoiesis suggests that these proteins may regulate the growth and development of trophoblasts or embryonic stem cells.

In the mouse, the formation of blood cells, or hemopoiesis, begins in the yolk sac at approximately day 7 of embryogenesis. The major site of hemopoiesis shifts to the fetal liver by day 10 of gestation and finally to the bone marrow, which remains the major hemopoietic organ in the adult (17). Soluble molecules such as interleukin-1 through interleukin-7 (IL-1 through IL-7) and colony-stimulating factors (granulocyte-macrophage colony-stimulating factor [GM-CSF], granulocyte CSF, and macrophage CSF) play an important role in regulating the growth and differentiation of various hemopoietic lineages in the adult animal. In particular, IL-3, IL-6, and GM-CSF, which have multiple biological effects on mature cells, also appear to act on early hemopoietic progenitor cells (10, 21, 25, 28). Leukemia inhibitory factor (LIF), on the other hand, has no reported effects on normal bone marrow progenitor cells but shows some similarities to IL-6 in other functions, such as the induction of differentiation of myeloid leukemic cells (7). In addition, it has recently been shown that LIF maintains the in vitro growth of embryonic stem cells derived from the inner cell mass of blastocysts (24, 27) and therefore might be active in the blastocyst in vivo. We believed it of interest to assess the expression of IL-3, IL-6, LIF, and GM-CSF in the blastocyst prior to developmental commitment as a first step in determining whether these molecules play a role very early in embryogenesis.

Blastocysts express mRNA for LIF and IL-6. Blastocysts were individually isolated either directly from timed pregnant C57BL/6 mice or by culturing two-cell embryos to the blastocyst stage (9; Fig. 1) and subjected to extensive washing in medium. RNA was isolated from blastocysts (19), reverse transcribed (13), and subjected to 40 cycles of polymerase chain reaction (PCR) amplification (thermal cycling times: 94°C, 1 min; 55°C, 2 min; 72°C, 3 min), using pairs of oligonucleotide primers specific for IL-3, IL-6, GM-CSF, and LIF. The sequences of the primers and the sizes of their predicted PCR products are shown in Table 1. In addition, we estimated the sensitivity of target sequence detection by using each set of primers. Plasmids containing cDNA inserts for IL-3, IL-6, LIF, and GM-CSF were serially diluted and used as target sequences for each PCR. The limits of detection were assayed by Southern blot analysis, using ^{32}P-labeled cDNA probes. IL-3, LIF, and GM-CSF target sequences were detected at levels of approximately 0.032 fg and greater. This endpoint corresponds to approximately 10 molecules. IL-6 PCR assays were slightly less sensitive, detecting approximately 0.16 fg of plasmid target sequence (data not shown).

Blastocyst, positive control, and negative control PCR products for IL-3, IL-6, LIF, and GM-CSF were fractionated on agarose gels, and specific amplified fragments for IL-6 and LIF were detected in some but not all experiments by ethidium bromide staining. However, hybridization of Southern blots with the appropriate cDNA probes (washed to a final stringency of 0.1× SSC [SSC is 0.15 M NaCl plus 0.015 sodium citrate]–0.1% sodium dodecyl sulfate at 65°C) reproducibly revealed a specific signal for both IL-6 and LIF. Signals for IL-3 and GM-CSF were not found (Fig. 2). The IL-6 and LIF signals were specific to embryo-derived RNA. As a control, the medium in which blastocysts were rinsed was treated as a parallel sample. No hybridization signals were present, indicating the absence of somatic cell contamination from uterine wall cells (Fig. 2, lanes 1). Moreover, the signals could not be due to contamination by genomic sequences for IL-6 and LIF in the RNA preparation since the PCR primers were designed to span introns and would produce a larger amplification product (Fig. 2B, lane 2). Identical results for each gene were obtained in at least two independent experiments. In at least one experiment, all four genes were analyzed from the same preparation of blastocyst mRNA and reverse-transcribed products. Thus, at similar levels of sensitivity in the PCR, we conclude that both IL-6 and LIF genes are transcribed in blastocysts, whereas IL-3 and GM-CSF mRNA were not detectable. This specificity in the pattern of expression implies that blastocysts do not indiscriminately transcribe hemopoietic regulatory factor genes.

Cultured blastocysts secrete IL-6 and LIF activities. To determine whether functional proteins were produced from

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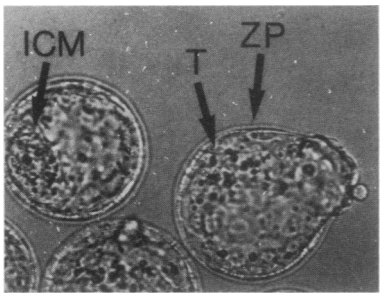

FIG. 1. Phase-contrast photomicrograph of C57BL/6 blastocysts (magnification, ×200). Two cell types are visible, trophoblasts (T) and the inner cell mass (ICM). At the top right, a blastocyst is hatching from the zona pellucida (ZP).

these transcripts, 150 to 200 fully expanded blastocysts were cultured in 200 μl of Dulbecco modified Eagle medium supplemented with 20% fetal bovine serum for an additional day. The supernatants were collected and assayed on IL-6- or LIF-dependent cell lines in at least two independent experiments (Fig. 3). Cultured blastocysts secreted an activity that supported the proliferation of an IL-6-dependent plasmacytoma, KD83, a derivative of MOPC 104E (23; Fig. 3A). This activity was specifically and completely blocked by a monoclonal antibody that neutralizes IL-6 biological activity (M. Pearce and J. Abrams, unpublished results). Functional LIF protein was assayed in a similar manner on DA1-a cells, a factor-dependent myeloid cell line that proliferates in response to certain factors, including LIF, IL-3, IL-4, and granulocyte CSF or GM-CSF, but is completely unresponsive to IL-6 (5; unpublished observations). Growth of DA1-a cells was supported by blastocyst supernatant (Fig. 3B). Since we do not have a neutralizing monoclonal antibody to LIF, we cannot specifically block this activity. However, using IL-3- and IL-4-dependent cell lines, we did not detect any bioactivity in blastocyst supernatant (data not shown), and no IL-3 or GM-CSF mRNA was detectable by PCR (Fig. 2). Others have reported the absence of granulocyte CSF mRNA in blastocysts (19). Thus, these results are consistent with the production of functional LIF protein by blastocysts.

Cultured blastocysts are still capable of developing into viable offspring. We showed above that IL-6- and LIF-specific RNA was present in fully expanded blastocysts isolated directly from uterine horns or developed in vitro from two cell embryos but that functional proteins were not detectable until after an additional day of culture. This period of time may be necessary for the accumulation of

TABLE 1. PCR primers for detection of hemopoietic regulatory factor gene expression

Gene	Primer location (bp)[a]		Expected size (bp)[b]	Expected genomic size (bp)[c]
	5′	3′		
IL-3	286–310	555–578	292	549
IL-6	26–44	481–505	479	4,869
LIF	1–28	628–653	653	1,300
GM-CSF	222–246	511–535	313	1,812

[a] Numbers indicate the location on the cDNA sequence, as described previously (3, 8, 15, 16).

[b] Of the amplification product generated from a cDNA from blastocyst mRNA.

[c] Of an amplification product derived from genomic DNA, as described previously (15, 16, 26; F. Conquet and P. Brulet, personal communication).

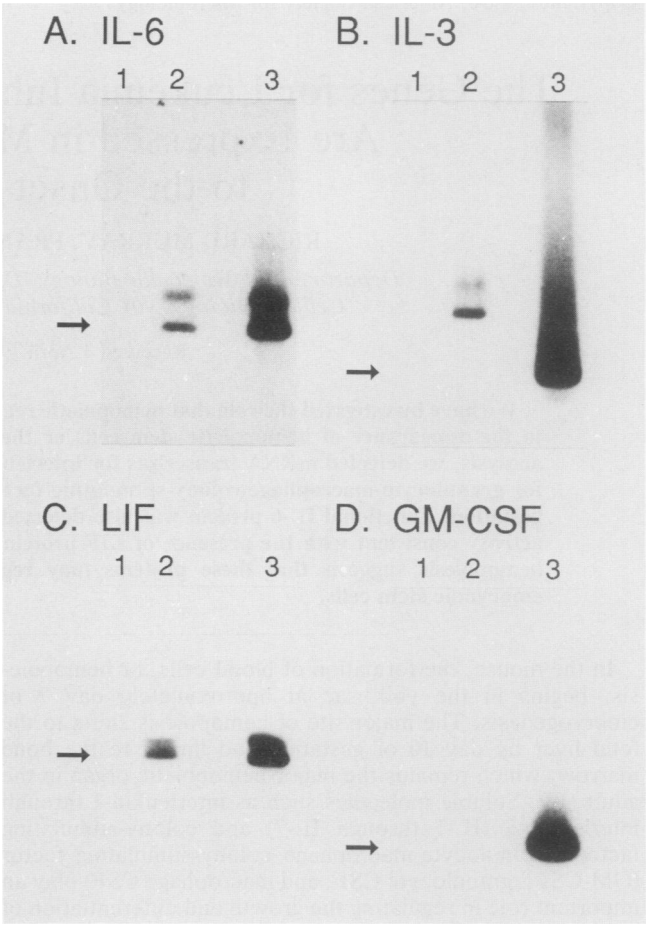

FIG. 2. Hemopoietic factor gene expression in blastocysts. Southern blot filters of PCR amplification products were hybridized with ^{32}P-labeled cDNAs for IL-6 (A), IL-3 (B), LIF (C), and GM-CSF (D). The samples from each panel are rinse media (lane 1), blastocysts (lane 2), and a concanavalin A-stimulated T-cell clone, D10 (lane 3). Each PCR reaction for blastocysts contained cDNA from the mRNA of approximately 12 embryos, and those for D10 contained cDNA from 10 pg of total RNA. Results for IL-3, IL-6, and GM-CSF PCR were from the same preparation of blastocyst mRNA, whereas the PCR of LIF was from a different preparation. All samples were from blastocysts developed in vitro from two-cell embryos. Identical results have been observed for IL-3, IL-6, and GM-CSF from freshly isolated blastocysts. Arrows indicate the expected size of amplified products from cDNA. In addition to the appropriate-size band indicative of IL-6 and LIF expression, we consistently observed a longer amplification product for both IL-6 and LIF in the blastocyst and the D10 positive control cDNA preparations. This band is also present when a defined plasmid template is used as the target sequence.

blastocyst-derived products. During this time the embryos have also hatched from the zona pellucida (Fig. 1), which may enhance detection of IL-6 and LIF proteins. These same hatched blastocysts were still capable of forming viable offspring when implanted into surrogate animals (data not shown), indicating that no significant abnormalities occurred during hatching in vitro. We believe that the expression of IL-6 and LIF mRNA from fully expanded blastocysts, secretion of biologically active protein from hatched blastocysts, and the ability of the hatched blastocysts to form normal offspring indicate that IL-6 and LIF production is a

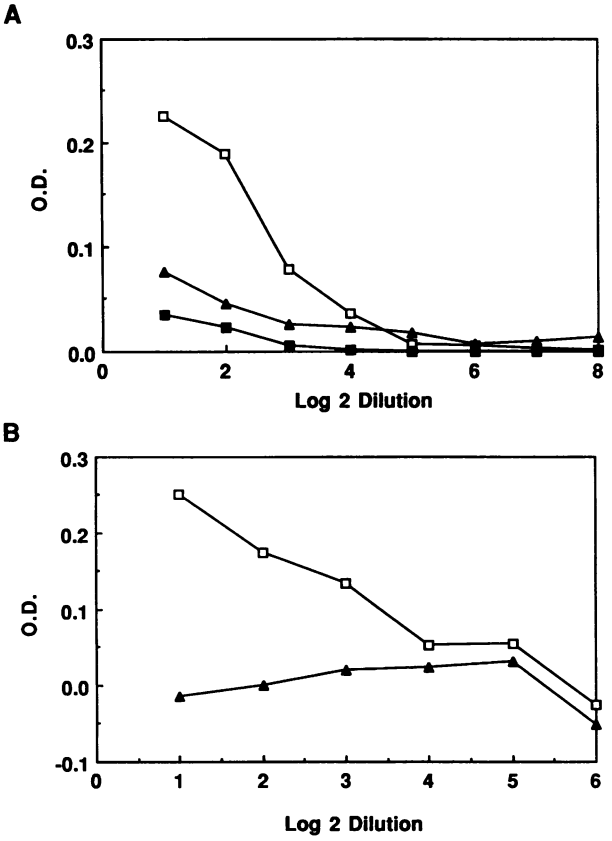

FIG. 3. (A) Secretion of IL-6 protein by cultured blastocysts. Two-cell embryos were isolated and washed extensively before culturing to the blastocyst stage; 150 to 200 blastocysts were then incubated in 200 μl of Dulbecco modified Eagle medium plus 20% fetal bovine serum for 1 day. Supernatants were assayed for bioactivity on IL-6-dependent KD83 cells (5,000 cells per well) in the presence (■) or absence (□) of neutralizing anti-IL-6 antibody. After 2 days, the number of viable KD83 cells was determined by the MTT colorimetric assay and expressed as optical density (O.D.) (18). The starting dilution represents a 1/2 dilution of the supernatant. The background was determined by culturing KD83 cells in medium alone (▲). (B) Secretion of LIF by cultured blastocysts. LIF was assayed on DA1-a cells in a manner similar to that used for IL-6. The cells (20,000 cells per well) were cultured with blastocyst supernatant (□) or with medium alone (▲) for 3 days. The starting dilution represents 1/4 dilution of the supernatant.

normal physiological process prior to or during implantation in vivo.

The data presented here document the first detection of hemopoietic regulatory factor gene expression in mouse preimplantation embryos, even in the absence of exogenous stimulation. This finding broadens the potential role of these molecules to include developmental and proliferative functions prior to differentiation of totipotent embryonic stem cells. Because most maternal mRNAs are thought to be degraded at the two-cell embryo stage (4, 12), it seems unlikely that the transcripts and proteins for IL-6 and LIF detected in blastocysts are maternal in origin.

Rappolee et al. have described the expression of transforming growth factors β1 and α and of platelet-derived growth factor A in preimplantation embryos (19). Teratocarcinoma cells, presumed to be analogous to primitive ectoderm, are also capable of growth factor production (11). However, studies of hemopoietic regulatory molecules in embryonic development have typically focused at mid-gestational stages and beyond, following the onset of hemopoiesis. For example, CSF-1 and its receptor, c-*fms*, have been shown to be expressed maternally at 7 days and in embryonic tissue at 9.5 days of gestation (20). Others have studied the expression of erythroid developmental factors in yolk sacs (14), again well after the onset of hemopoiesis.

Our results show that both IL-6 and LIF genes, but not IL-3 or GM-CSF genes, are expressed in blastocysts in vivo. IL-6 and LIF share a number of functional similarities with each other. It has previously been shown that both IL-6 and LIF can induce myeloid cell differentiation in vitro (2, 7) and neuronal differentiation in vitro (22, 29). In addition, IL-6 and LIF regulate other cellular processes, including inflammatory and acute-phase responses (1, 3, 6). Thus, the coordinate expression of IL-6 and LIF genes in blastocysts further extends the similarities between these two molecules. Combined with previous reports that recombinant LIF maintains the totipotency of embryonic stem cells in vitro (24, 27), it is possible that both of these secreted molecules play an important role very early in development, perhaps in regulating proliferation and differentiation of uncommitted cells of the inner cell mass or in the development of the first hemopoietic stem cells. In addition, the ability of viable hatched embryos to actively secrete IL-6 and LIF implies that another target of their biological actions in vivo may be maternal uterine wall tissue. These cells, in response to IL-6, LIF, or both, may in turn regulate other processes necessary for the effective postimplantation development of the fetus.

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