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To the editor:

A potential role for leukemia inhibitory factor in the increased clonogenicity of human fetal progenitor cells

A recent report by Shih et al demonstrates the ex vivo expansion of human fetal $CD34^+$, thy-1^+ hematopoietic progenitors in the presence of a leukemia inhibitory factor (LIF)–induced, murine stromal cell–derived factor.[1] The authors show that expanded progenitor cells retain their immature immunophenotype in vitro and that the engraftment capacity in murine transplantation models is preserved. In this and their previous report, they allude to the potential utility of such an expansion promoting factor (SCEPF) in the clinical arena, including the transplantation of stem cells from cord blood.[1, 2]

In their experiments, a given LIF concentration of 10 ng/mL results in the production of a LIF-induced expansion promoting factor by the murine stromal cell line AC 6.21. They do not report how this concentration was determined to be optimal, nor do they report whether a dose response relationship between LIF concentration in AC 6.21 stromal cell culture and the magnitude of expansion was observed. The experimental design did not seem to address a potential direct effect of human LIF, in the dose range used, on $CD34^+$, thy-1^+ progenitors in culture, as control studies with LIF-neutralizing antibody were not conducted.

Based on our own experiments, levels of LIF protein in unstimulated adult peripheral ($n = 6$) and neonatal umbilical cord blood ($n = 13$) are at the limit of detection when measured by enzyme linked immunoadsorbant assay (ELISA) and are, therefore, several orders of magnitude below the concentration used by the authors. A significant rise in LIF protein levels, however, can be effected by stimulation of mononuclear cells, especially those derived from cord blood. Combined activation with anti-CD3 and IL-2 leads to a more than 20-fold increase in LIF production by cord blood mononuclear cells, compared to a 7-fold increase seen with adult PBMC (Figure). This effect is blunted (4- and 5-fold increase, respectively) after monocyte (CD14) depletion, suggesting that T cell–monocyte interaction is required. Stimulation with interferon γ, tumor necrosis factor α, interleukin-1β, or lipopolysacharide results in moderate 2- to 3-fold increased LIF production by cord blood derived mononuclear cells.

Other groups have demonstrated the markedly increased clonogenicity of cord blood-derived progenitors and its expansion potential upon addition of LIF to culture conditions.[3, 4] At least in the murine model, LIF-induced expansion of progenitors appears to be based on the increased production of more primitive progenitors with greater replicative potential.[5]

We therefore wish to suggest that the expansion effect described by Shih et al may rely on the supraphysiologic levels of LIF in their stromal cell culture system that in turn lead to the elaboration of an expansion promoting activity. Moreover, it remains to be determined, whether in addition to the induction of SCEPF in murine stroma, human LIF used at nanogram concentrations has an independent effect on the expansion of human fetal progenitors. Taken together, the augmented production of LIF by cord blood mononuclear cells may directly and/or indirectly underly the increased in vitro and in vivo clonogenicity of cord blood, compared to adult PBMC.

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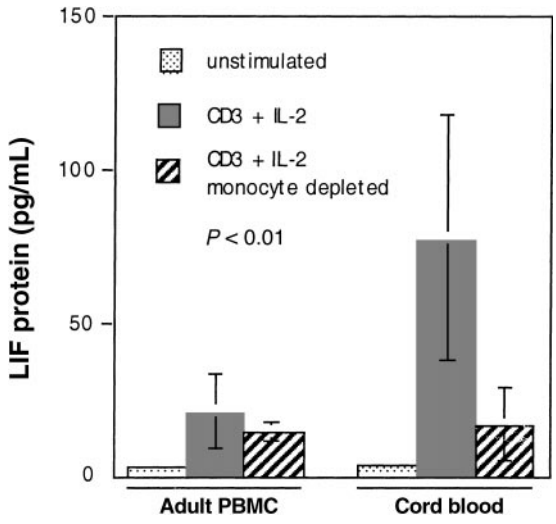
Supported by grants from the Deutsche Krebshilfe (W 11/94 Bu2) and the Elterninitiative Kinderkrebsklinik eV.



Levels of LIF protein measured by ELISA. Stimulation with mouse anti-CD3 monoclonal antibody and interleukin-2 was carried out in liquid culture over 72 hours.

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Response:

Leukemia inhibitory factor does not have a direct effect on the expansion of transplantable human hematopoietic stem cells

We thank Drs Kurre and Burdach for their interesting comments about the possibility that leukemia inhibitory factor (LIF) might have a direct effect on the expansion of human fetal progenitor cells. We would like to take this opportunity to address those concerns that have been raised by Drs Kurre and Burdach:

1. Whether a dose response relationship between LIF concentration in AC6.21 stromal cell culture and the magnitude of expansion was observed. Before we began to collect hundreds of liters of stromal conditioned medium from LIF-treated cultures (SCM-LIF) for protein purification, we asked ourselves whether the amount of LIF in the stromal cell culture could be reduced without losing the stem-cell-expansion promoting factor (SCEPF) activity. Experiments were performed in our laboratory to determine the minimal concentration of LIF for the production of SCEPF. SCM-LIFs were prepared from stromal cell cultures treated with various amounts of LIF and assayed for SCEPF activity as previously described.[1,2] Our results demonstrate that there is a dose response relationship between LIF concentration in AC6.21 stromal cell culture and the magnitude of expansion (Table). There is no detectable SCEPF activity in the SCM-LIFs when the stromal cell cultures were treated with less than 50 pg/mL of LIF. SCEPF activity in the SCM-LIFs is proportionally increased when the concentration of LIF in the stromal cell cultures is increased from 50 pg/mL to 500 pg/mL. The SCEPF activity in the SCM-LIFs is saturated when the concentration of LIF is higher than 500 pg/mL. We now routinely use 500 pg/mL as the optimal dose of LIF in our stromal-based culture system and for preparation of SCM-LIFs for SCEPF protein purification.

Dose response of LIF on the production of SCEPF by stromal cells

LIF Doses in Stromal Cultures	Frequency of $CD34^+$ thy-1^+/Positive Wells*	Percentage of $CD34^+$ Thy-1^+ Cells†
10 ng	100% (10/10)	15 ± 3
5 ng	100% (10/10)	15 ± 4
2.5 ng	100% (10/10)	15 ± 5
1 ng	100% (10/10)	15 ± 4
500 pg	100% (10/10)	14 ± 5
250 pg	60% (6/10)	8 ± 3
100 pg	20% (2/10)	3.6 and 2.6
50 pg	0% (0/10)	N/A
25 pg	0% (0/10)	N/A
10 pg	0% (0/10)	N/A

Stromal conditioned mediums (SCMs) were prepared from stromal cultures treated with various doses of LIF. Preparation of SCM and the assay for the SCEPF activity in the SCMs were performed as previously described.[1,2]

*A well is scored as $CD34^+$ thy-1^+/positive only if it has detectable (>1%) of $CD34^+$ thy-1^+ cells in the well. Numbers in parentheses indicate the total number of $CD34^+$ thy-1^+/positive wells divided by the total number of wells analyzed in the experiments.

†Data is presented as the mean ± SD of the total number of $CD34^+$ thy-1^+/positive wells in each culture condition. N/A indicates not applicable.

2. Whether there is a potential direct effect of human LIF, in the dose range used (10 ng/mL), on $CD34^+$ thy-1^+ progenitors in culture. We have 3 sets of data that argue against the notion that LIF might have a direct effect on $CD34^+$ thy-1^+ progenitors. First, as we have shown in a previous report,[1] there is no significant difference in the ability to support proliferation and differentiation of $CD34^+$ thy-1^+ progenitors between the stromal cultures treated with either 10 ng/mL of human LIF or murine LIF, the latter of which cannot bind to the human LIF receptor. This result demonstrates that the action of LIF is indirect and mediated via the stromal cells to facilitate ex vivo expansion of transplantable human fetal BM $CD34^+$ thy-1^+ cells. Since $CD34^+$ thy-1^+ cells respond very similarly to both human LIF (which can bind to human and mouse LIF receptor) and murine LIF, suggesting that human LIF does not directly have any significant effect on $CD34^+$ thy-1^+ cells proliferation and differentiation. Second, as we have shown in another report,[2] cells with $CD34^+$ thy-1^+ phenotype can be detected only in the positive control culture (treated with 200% SCM-LIF) and not in any other culture conditions treated with 200% stromal conditioned medium from cultures in the absence of LIF (SCM), 10 ng/mL LIF, and various combinations of cytokines including interleukin-3, interleukin-6, granulocyte macrophage colony-stimulating factor, stem cell factor, flt-3 ligand, and thrombopoietin. These results further suggest that LIF does not have a direct effect on expansion of human fetal $CD34^+$ thy-1^+ cells even at concentrations as high as 10 ng/mL. Third, to further rule out a direct role of LIF in facilitating ex vivo stem cell expansion, control studies with neutralizing antibody against LIF were conducted. Our data demonstrated that the production of murine LIF is upregulated by the stromal cells in the presence of 10 ng/mL of either recombinant human or murine LIF (data not shown). Although the concentration of murine LIF was not detectable by ELISA (R&D Systems, Minneapolis, MN; sensitivity is 50 pg/mL) in either SCM or SCM-LIF, we were able to demonstrate a 3-fold increase in LIF expression in the LIF-treated AC6.21 cells by RT-PCR (data not shown). SCM-LIF was prepared from stromal cultures treated with 500 pg/mL recombinant human LIF (R&D Systems). The concentration of residual human LIF in the SCM-LIF was then determined to be in the range of 200-250 pg/mL by ELISA. $CD34^+$ thy-1^+ cells purified from human fetal BM were cultured on 200% SCM-LIF for 3 weeks in the absence or presence of 0.1 to 10 μg/mL of neutralizing antibody against human LIF (R&D Systems) as previously described.[2] We found similar results, including 100% of wells (20 of 20) that were $CD34^+$ thy-1^+/positive and an average of 9% (9 2) of $CD34^+$ thy-1^+ cells in each well, for all cultures including positive control (200% SCM-LIF without neutralizing antibody) (data not shown). This result shows that ex vivo stem cell expansion was not affected by the addition of various concentrations (0.1, 1,

and 10 μg/mL) of neutralizing antibody against human LIF to the cultures in both the frequency of $CD34^+$ thy-1^+/positive wells and the percentage of $CD34^+$ thy-1^+ cells in the wells. This result demonstrates that neutralizing antibody against LIF cannot block the ex vivo stem cell expansion facilitated by the SCEPF activity in the SCM-LIF and suggests that LIF does not contribute directly to the SCEPF activity in the SCM-LIF. Taken together, our results do not support the notion that LIF might have a direct role in facilitating ex vivo expansion of hematopoietic stem cells.

3. Whether the augmented production of LIF by cord blood (CB) mononuclear cells may directly and/or indirectly underly the increased in vitro and in vivo clonogenicity of CB, as compared to adult peripheral blood mononuclear cells (PBMC). Drs Kurre and Burdach have presented their data to demonstrate that there is a higher production (2- to 3-fold) of LIF by the CB mononuclear cells than the adult PBMC during stimulation from interferon γ, tumor necrosis factor α, interleukin-1β, or lipopolysaccharide. Combined activation with anti-CD3 and interleukin-2 leads to a more than 20-fold increase in LIF production by CB mononuclear cells, compared to a 7-fold increase seen with adult PBMC. This effect is blunted (4- and 5-fold increase, respectively) after monocyte (CD14) depletion, suggesting that T-cell–monocyte interaction is required. It is interesting to note the correlation of their data with several previously published reports, suggesting that LIF might involve expansion of more primitive progenitors with greater replicative potential.[3-5] But many lines of evidence do not support this notion. First, many studies have revealed ontogeny-associated differences in a variety of functional attributes of stem-cell proliferation and differentiation in both in vivo and in vitro settings.[6-8] It has been recently demonstrated that long-term engrafting cells were approximately enriched in CB $CD34^+$ cells 8-fold more than in adult PBMC $CD34^+$ cells, and each CB long-term engrafting cell had an approximately 15-fold higher multilineage proliferative capacity.[8] Thus these results suggest that an intrinsic qualitative difference might be responsible for the remarkable difference between these 2 sources of stem/progenitor cells.[7,8] Second, LIF is a pleiotropic cytokine with distinct hematopoietic activities. In vivo treatment of mice with recombinant murine LIF induces thrombocytosis and increases the number of hematopoietic progenitor cells in spleen and bone marrow.[9] It has been further determined that in vivo treatment with LIF expands the number of committed progenitor cells and BM-repopulating cells that accelerate short-term hematopoietic reconstitution without increasing radioprotection.[10] These data do not support a direct role for LIF as a single factor for promoting expansion of hematopoietic stem cells in vivo. Third, it is generally believed that production of many cytokines other than LIF will be increased by CB mononuclear cells with various stimulation. To prove the concept that the augmented production of LIF by CB mononuclear cells may directly and/or indirectly underly the increased in vitro and in vivo clonogenicity of CB, as compared to adult PBMC, it will be required to establish the panel of cytokines whose production have been up-regulated and to rule in or rule out the contribution of these cytokines including LIF for the activity to increase in vitro and in vivo clonogenicity of CB mononuclear cells.

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Supported by National Cancer Institute grants NCI PPG CA 30206, NCI CA 33572, and NCI CA 71866.

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