Vol. 199, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1994 Pages 220-226

Recombinant human leukemia inhibitory factor is mitogenic for human bone-derived osteoblast-like cells

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Received January 3, 1994

Recombinant human leukemia inhibitory factor (rhLIF) is mitogenic for human bone-derived osteoblast-like cells in vitro. [^{3}H]-thymidine incorporation into DNA was stimulated dose-dependently in a prostaglandin E_2-independent manner. rhLIF exerted no effect on either the basal or $1,25(OH)_2D_3$-induced synthesis of osteocalcin or alkaline phosphatase (AP) activity. Following treatment with rhLIF, radiolabelled cell nuclei were co-localized with AP, indicating that the mitogenic effect of rhLIF occurs on cells of the osteogenic lineage. Local generation of LIF at the human bone surface may therefore serve as a potential autocrine/paracrine mitogen for progenitor cells of the osteoblastic lineage and so could correlate with the known bone forming properties of LIF.

Evidence implicates osteoblast-derived growth factors and cytokines in the localized regulation of bone cell function and bone remodelling (1). Recently, the role of leukemia inhibitory factor [LIF] in bone metabolism has been described. LIF has a pleiotropic spectrum of action on a variety of tissues (2), including its effects on bone. LIF can both stimulate bone resorption by mechanisms that involve the stimulation of osteoclast formation and function (3-5), as well inhibiting basal bone resorption (6). LIF receptors have been detected on osteoblast-like cells (obs) but not on osteoclasts (7-9). In vitro studies indicate that obs are modulated by LIF. In organ culture, LIF stimulates cell proliferation and protein synthesis (5). The effects of LIF on isolated obs are complex with cell growth, enzyme and bone matrix protein synthesis being regulated (8-10, 11, 12). Bone is a target tissue for LIF in vivo. Following injection of murine hematopoietic FDC-P1 cells transfected with a retroviral construct containing the cDNA encoding LIF into mice (7), over expression of LIF induced the development of a fatal syndrome involving multiple organs including the stimulation of extensive new trabecular bone formation. However, systemic injection of LIF did not similarly induce new trabeculae formation although alterations in cortical bone were observed (13). LIF is produced by a variety of cell types (2) including human obs (14), clonal murine MC3T3-E1 (4,15) and normal and malignant rat obs in culture (9). In this study we report on the effects of rhLIF on human obs to determine the potential for an autocrine/paracrine control of human bone cell activity.

0006-291X/94 $5.00

MATERIALS AND METHODS

Materials

rhLIF was expressed in CHO cells and purified within Sandoz Pharma AG, Basel. The half maximal stimulation of the LIF responsive DA-1a cell line occurs at at concentration of 2ng/ml. Tissue culture materials were purchased from Gibco Ltd. Recombinant human interleukin-1β (rhIL-1β) (5 x 10^8 U/mg) was expressed in *E. coli* and purified within Sandoz Pharma AG, Basel. 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) was a gift from Dr M.R. Uskokovic, Hoffmann-La Roche Inc., USA.

Human bone cell culture

Explants of human trabecular bone were cultured as described previously (16). All experimental incubations unless stated were conducted in EMEM containing 0.1% (w/v) BSA, penicillin-streptomycin (100 U/ml - 100 μg/ml), L-ascorbate (50 μg/ml), vitamin K (10^{-8}M) and L-glutamine (2mM). Where the influence of serum on the response of rhLIF was examined, charcoal-strippped FCS (CSFCS) was used and prepared as described previously (16).

Cell proliferation: radiolabeled thymidine incorporation

Cell proliferation was assessed by the incorporation of [^{3}H]-thymidine into trichloroacetic acid precipitable materials as described previously in detail (16).

Assay of osteocalcin, alkaline phosphatase, prostaglandin E_2 and cell protein

Assays of osteocalcin, alkaline phosphatase (AP), cell protein and prostaglandin E_2 (PGE_2) were conducted as described previously (16). Osteocalcin was measured by commercial radioimmunoassay (BTI, USA).

AP histochemistry and autoradiography

For the histochemical localization of AP with radiolabelled cell nuclei, a cell labelling procedure similar to that described by Rickard et al was used (17). Cells grown on multi-chambered slides were incubated in the presence or absence of rhLIF (1000ng/ml) for 48h and during the last 24h of this incubation period with [^{3}H]-thymidine. The cells were washed in PBS to remove un-incorporated [^{3}H]-thymidine and new culture media containing 10^{-8} M $1,25(OH)_2D_3$ was added for 72h to induce AP expression. Cells were then fixed in situ with 4% (v/v) paraformaldehyde followed by a thorough wash in PBS. AP was then visualized on the fixed cell layer as described previously using Fast red salt (18). Cells were then coated in Kodak NTB 3 liquid autoradiography emulsion, allowed to dry and then exposed for 6 days prior to development.

Statistics

Statistical differences between treatments were determined using analysis of variance.

RESULTS

Human bone cell proliferation was stimulated by rhLIF dose-dependently (10-1000 ng/ml) (Fig. 1a). This effect was most pronounced at 1000 ng/ml, whilst concentrations below 10 ng/ml had no detectable effect. No changes in the acid soluble pool were observed (data not shown). The mitogenic effect of rhLIF (1000 ng/ml) was increased in the presence of serum, indicating an interaction with serum components (Fig. 1b). However, no alterations in the stimulatory dose response profile were observed in the presence of 5% (v/v) CSFCS (data not shown). The proliferative effect of rhLIF occurred in a PGE_2-independent manner, as assessed by co-incubation with indomethacin (10^{-6} M) (Fig. 1c). Measurements of media PGE_2 confirmed that rhLIF (0.1-1000 ng/ml) had no stimulatory effect on PGE_2 synthesis (data not shown). In contrast, rhIL-1β (1000 U/ml) stimulated PGE_2 synthesis (Basal PGE_2 levels = no detectable production: rhIL-1β = 41.5 pg PGE_2/ug protein) in the same experiment.

Treatment of human obs with rhLIF (0.1-1000 ng/ml) had no effect on the basal or $1,25(OH)_2D_3$-induced AP activity (Fig. 2a). However in one culture, a potentiation of the $1,25(OH)_2D_3$-induced AP activity was observed with 1000 ng/ml rhLIF (Fig. 2a insert). rhLIF

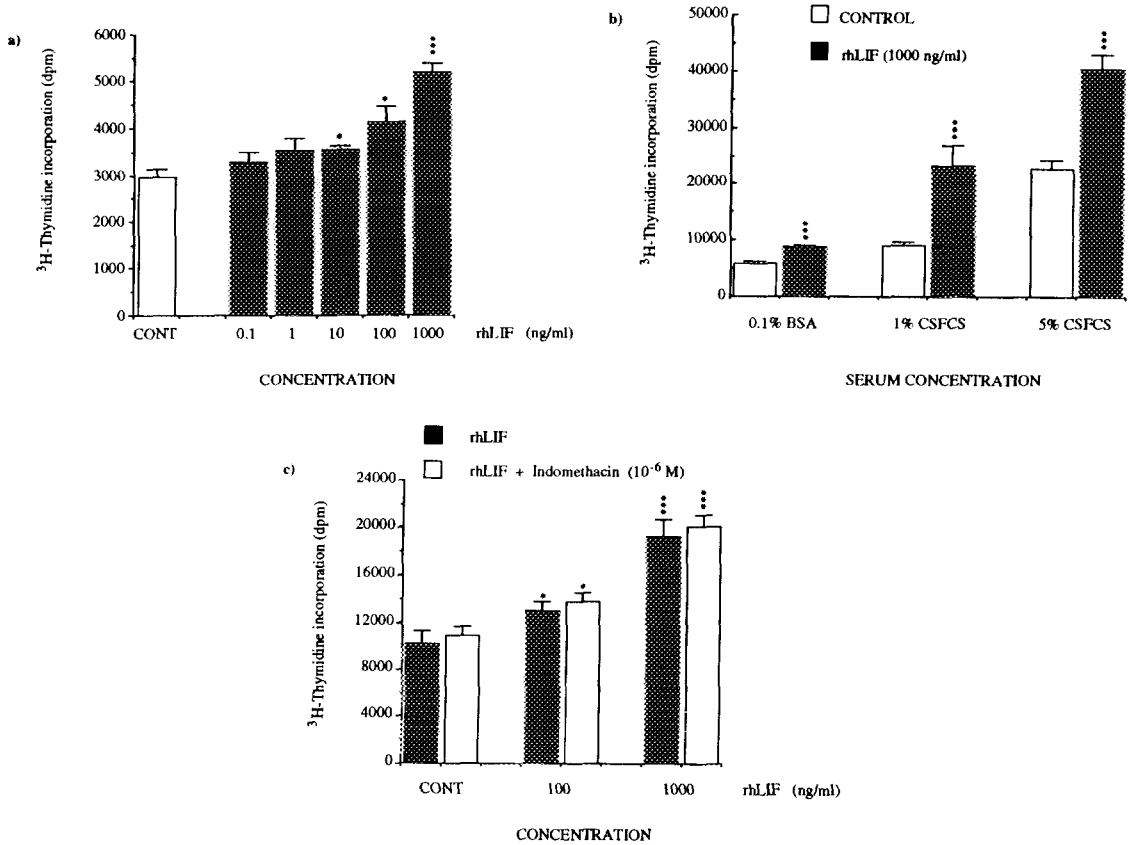

Fig. 1. **Stimulation of cell proliferation of human osteoblast-like cells by rhLIF.** a) Under serum-free (0.1% (w/v) BSA) conditions. b) Influence of serum. c) Influence of indomethacin. Cell proliferation was assessed by [^{3}H]-thymidine incorporation as described in Materials and Methods. Values represent mean ± S.E.M. (n=4). Significant difference from control, * $p < 0.05$, *** $p < 0.001$.

(0.1-1000 ng/ml) had no measurable effect on osteocalcin production by the human obs either when added alone or following induction by $1,25(OH)_2D_3$ (Fig. 2b). Comparison of the actions of rhLIF (1000 ng/ml) and rhIL-1β (100 U/ml) in the presence of 5% (v/v) CSFCS demonstrated that, unlike rhLIF, rhIL-1β (100 U/ml) decreased the osteocalcin production stimulated by $1,25(OH)_2D_3$ (Fig. 2b insert).

The predominant effect of rhLIF on the human obs is clearly a mitogenic response with, in certain circumstances, an enhancement of AP expression. To determine whether the mitogenic effect of rhLIF was potentially exerted on osteoprogenitor cells, the influence of rhLIF (1000 ng/ml) on cell proliferation followed by subsequent induction with $1,25(OH)_2D_3$ of the expression of AP, a marker of osteogenic cells (19), was examined by dual autoradiography-histochemical localization. Our studies show that it was possible to co-localize these two parameters indicating that the proliferative effect is likely exerted on cells of the osteogenic lineage (Fig. 3).

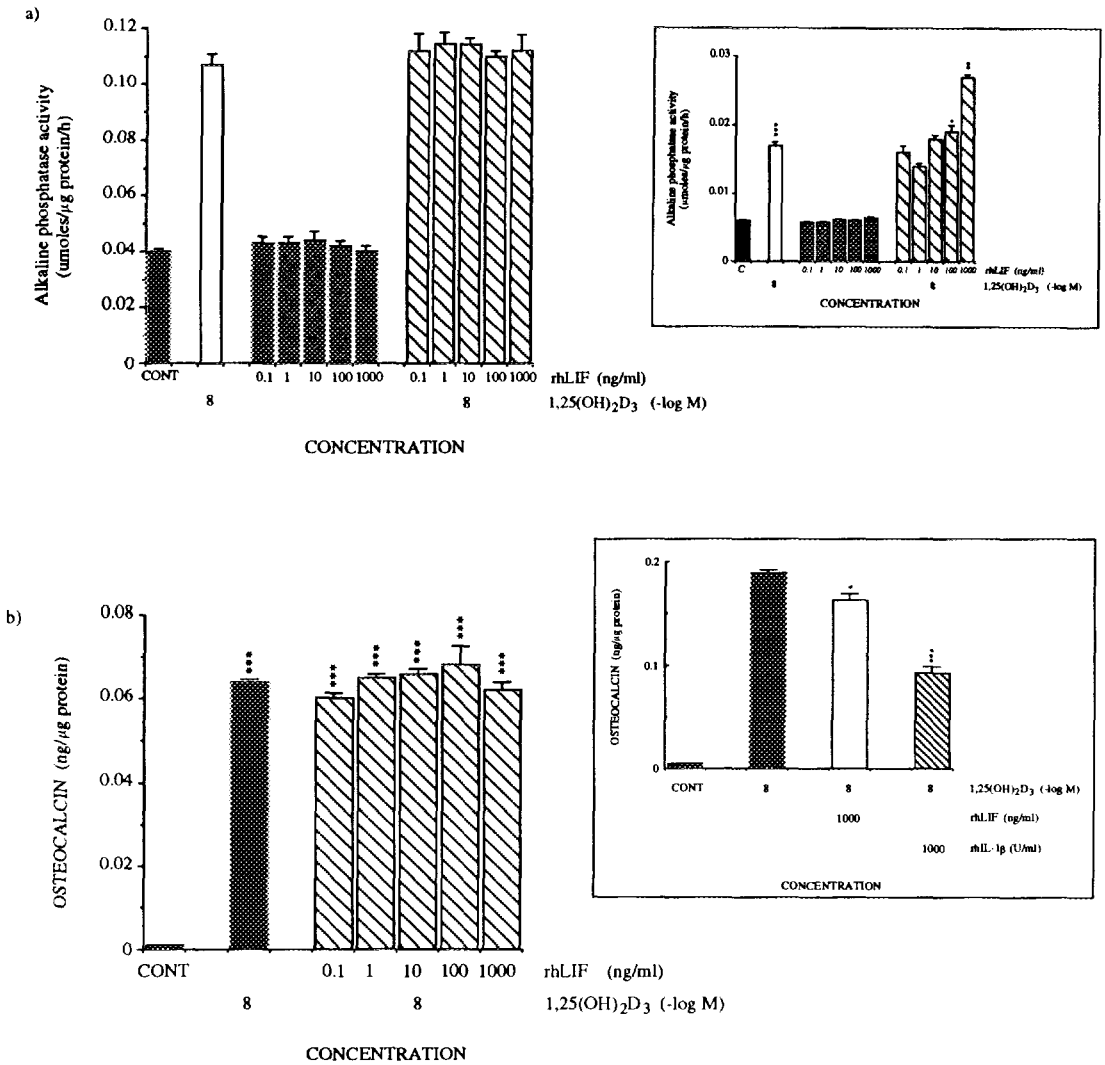

Fig. 2. **The influence of rhLIF on the basal and 1,25(OH)$_2$D$_3$-stimulated a) alkaline phosphatase activity and b) osteocalcin synthesis of human osteoblast-like cells.** a) The alkaline phosphatase activity of the solubilized cell layer was measured as described in Materials and Methods. Significant difference from control,***$p < 0.001$. Significant difference from 1,25(OH)$_2$D3-treated cells, ° $p < 0.05$, °° $p < 0.001$. b)The comparative studies of rhLIF and rhIL-1β were conducted in EMEM supplemented with 5% (v/v) CSFCS. The osteocalcin released into the culture medium was measured by specific radioimmunoassay as described in Materials and Methods. Significant difference from control, *** $p < 0.001$. (inset) Significant difference from 1,25(OH)$_2$D3-treated cells, * $p < 0.05$, *** $p < 0.001$.

DISCUSSION

We evaluated the effects of rhLIF on human obs and found that the predominant action of LIF on these cells is a stimulation of cell proliferation. Previously reported effects of LIF on obs proliferation in vitro are variable with stimulation being observed in isolated fetal rat obs, a decrease being detected in the

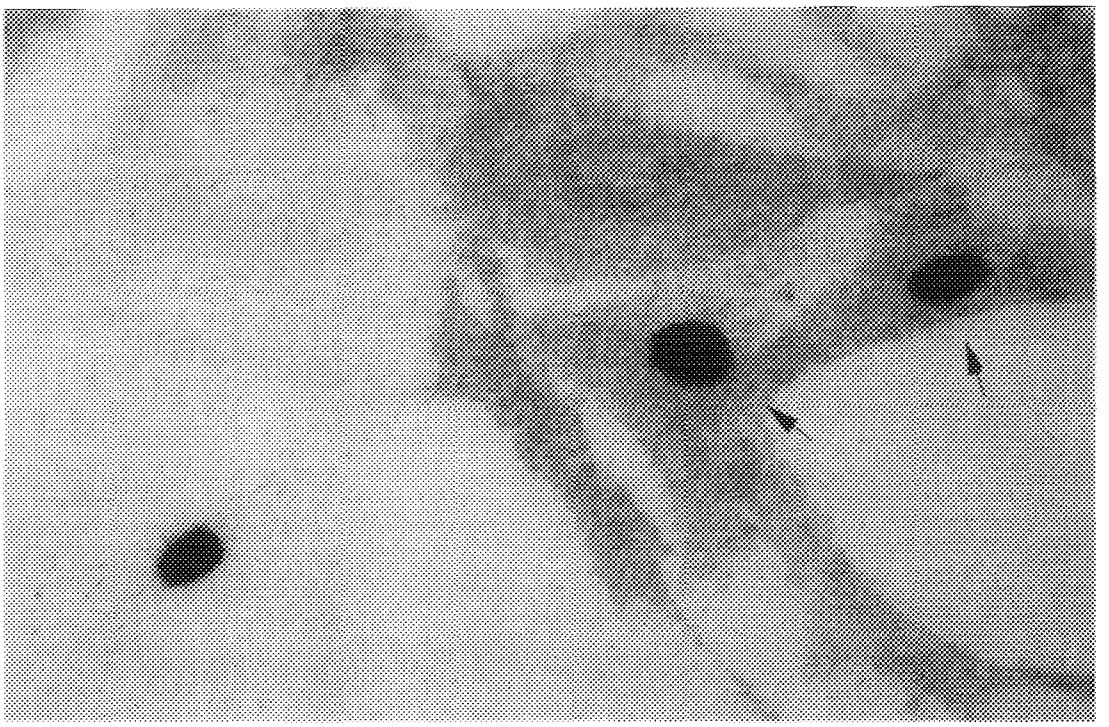

Fig. 3. **Co-localization of radiolabelled nuclei with AP activity.** Cells with co-localized radiolabelled nuclei and AP activity are indicated with an arrow.

murine osteogenic sarcoma cell line UMR-106 (11), whilst in the murine MC3T3-E1 cell line, LIF may either inhibit (10) or exert no effect on cell growth (12). In vivo, the local release of LIF within bone of mice engrafted with FDC-P1 cells expressing high levels of LIF markedly increased the number of obs leading to an increase in bone formation (7). In mouse calvarial organ culture, LIF stimulates cell proliferation following short-term culture whilst prolonged exposure with high LIF concentrations decreased cell proliferation. The effect of extended incubation with rhLIF on the human bone cells was not examined. Interestingly in neonatal mouse calvaria, a component of the stimulation of bone resorption by LIF appears to be dependent upon the mitogenic action of LIF (5,20). In our studies, rhLIF was found to be mitogenic for the human bone cells under a variety of culture conditions. The presence of serum enhanced the stimulation of DNA synthesisby rhLIF. Interestingly, the presence of serum did not alter the sensitivity of the cells to lower concentrations of rhLIF, as would likely be expected if this was related to some synergistic interaction with serum factors. It is of interest to note that the inhibitory effect of LIF on MC3T3-E1 cell proliferation (10) was reduced in high serum concentrations indicating that certain stimulators of growth were capable of counteracting the action of LIF. However, the influence of serum on the dose-responsiveness was not reported in the above study. The contribution of PGE_2 to the actions of LIF on obs is possible since PGE_2 mediates certain actions of some bone regulatory factors (21-24), including the stimulation of bone resorption by LIF (5). The stimulation of DNA synthesis by rhLIF in human obs was PGE_2-independent as judged by the co-culture with indomethacin and by direct measurement of media

PGE_2 levels following LIF treatment. The same cultures produce PGE_2 upon stimulation with IL-1β, indicating that the stimulation of PGE_2 synthesis does not represent a facet of rhLIF activity on human obs. Stimulation of DNA synthesis by LIF in mouse calvaria (5) and primary cultures of fetal rat calvaria cells (11) was unaffected by co-incubation with indomethacin. Although the media PGE_2 levels were not measured in these studies, these observations would also indicate that the stimulation of cell proliferation by LIF is PGE_2-independent.

Generally, no effect of rhLIF on the basal and $1,25(OH)_2D_3$-induced AP activity of the human obs was detected. However, in one culture where the $1,25(OH)_2D_3$-induced AP levels were low, a potentiation of the effect of $1,25(OH)_2D_3$ by rhLIF was detected which occured independently of its proliferative effect. Due to the lack of additional comparable donor cultures, this observation is preliminary and requires future clarification. In a similar manner to this observation, LIF has previously been reported to enhance the retinoic acid-induced synthesis of AP in the pre-osteoblastic clonal RCT-1 (8) and UMR-201 (9) cell lines. LIF may serve to both recruit osteoprogenitor cells and promote their differentiation depending on the presence of appropriate co-inducers and the status of cell differentiation of cells in the osteogenic lineage. In MC3T3-E1 cells, LIF decreases the basal AP activity via PGE_2-independent mechanisms (10,12) and also partially blocks the inhibitory effect exerted by $1,25(OH)_2D_3$ related to a decrease in $1,25(OH)_2D_3$ receptor number. Based on the modulation of $1,25(OH)_2D_3$-induced responses we found no evidence suggesting that similar mechanisms may exist in the human obs. Synthesis of bone matrix components is regulated by LIF. In MC3T3-E1 and RCT-1 cells, LIF suppresses type I collagen mRNA levels (8,10) whilst increasing osteopontin mRNA levels (10). In the present study rhLIF exerted no effect on osteocalcin synthesis either alone or following induction by $1,25(OH)_2D_3$. In the same experiments, rhIL-1β reduced the stimulatory effect of $1,25(OH)_2D_3$ on osteocalcin production, as observed previously (21, 25), indicating that both stimulatory and inhibitory regulatory mechanisms for this matrix protein exist in these cultures but are not influenced by LIF.

From the studies on the dual localization of radiolabelled nuclei with AP activity, this indicatess that the proliferative effect of rhLIF on human bone cells may be exerted on the osteoprogenitor cells within these cultures. The effect of IL-1 on proliferation of human bone obs is related to a comparable effect (17). Although several studies have identified that obs represent target cells for LIF, either by responsiveness (8-12) or by radio-ligand receptor localization (7-9), the mitogenic responsive cells within intact neonatal mouse calvaria have not been identified and due to the complex heterogenous nature of the intact bone, these may represent either osteogenic or non-osteogenic populations. The use of autoradiography in situ to identify histologically the mitogenic responsive cells may be of future interest to clarify this possibility.

In addition to representing target cells for the actions of LIF, obs have also been shown to produce LIF (3, 9, 12, 15), including human obs (14). Thus, LIF represents a potential autocrine/paracrine regulatory factor for human obs. In human obs the basal LIF production is stimulated by IL-1α, IL-1β, TNFα and $TGF\beta_1$ (14). Previously, IL-1 and TNF have been shown to be mitogenic for human osteoblast-like cells (17, 25-28). The present observations that LIF also stimulates the proliferation of the human obs highlights the possibility of the involvement of endogenous LIF generation by IL-1 and TNF being responsible, in part, for their mitogenic actions. Neutralizing antibodies are available to LIF which may be used to clarify if this is a functional possibility.

In conclusion, our studies have shown for the first time that LIF is mitogenic for cells in the human osteogenic lineage and coupled with our earlier observations that these cells also produce LIF (14), suggest

that the local generation of LIF at the surface of human bone could serve as an autocrine/paracrine mitogenic mechanism which may lead to the stimulation of new bone formation associated with increased osteoblast cell number (7, 29).

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