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Biphasic Effects of Leukemia Inhibitory Factor on Osteoblastic Differentiation

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Abstract Leukemia inhibitory factor (LIF) is a cytokine produced by multiple cell types including osteoblasts and which is active on bone metabolism. We have previously shown that in a bone nodule forming in vitro model of osteogenesis, the fetal rat calvaria (RC) cell model, LIF inhibits osteoblast differentiation, acting on late osteoprogenitors and/or early osteoblasts. These results are in contrast to in vivo experiments, in which LIF has been found to increase bone formation. To resolve this discrepancy, we have tested the effect of LIF on rat bone marrow (RBM) stromal cell cultures, an in vitro model encompassing earlier osteoprogenitor stages. LIF inhibited cell growth in early, proliferating RBM cultures, but increased the culture saturation density. The effect of LIF on bone nodule formation in this model was cell density dependent and biphasic. Continuous treatment with LIF reduced the number of bone nodules present in confluent, more mature cultures, and the inhibitory effect was strongest when cells were plated at higher cell density than lower. In contrast, during the early stages of RBM culture, nodule numbers were higher in LIF-treated dishes than in controls, and this effect was greater in lower density cultures. Acute LIF treatment restricted to early time points increased the final number of bone nodules formed in mature RBM cell cultures, but not in RC cell cultures. Our results indicate that LIF exerts complex, stage-specific effects on osteoprogenitor recruitment, differentiation, and bone formation, and that the effects are cell nonautonomous, in the rat bone marrow stromal cell model. J. Cell. Biochem. Suppl. 36:63–70, 2001.

Key words: leukemia inhibitory factor; bone nodules; colony assay; osteogenesis; differentiation; cytokines

Leukemia inhibitory factor (LIF) is a cytokine with multiple effects on bone. It has been found to increase bone turnover and bone tissue development in vivo, to induce a prostaglandin-dependent increase in bone resorption in organ cultures and to stimulate the proliferation of osteoblastic cells in vitro (reviewed in Martin et al., 1992; Malaval et al., 1998a). It is not clear, however, whether LIF has a direct effect on bone formation. We therefore tested its action in in vitro models of osteogenesis in which osteoblast differentiation and bone formation occurs in the absence of resorption. In fetal rat calvaria (RC) cell cultures, osteoprogenitors proliferate and differentiate to form bone nodules, the production of which can be quantified, constituting a colony assay for factors affecting osteoprogenitor differentiation and bone formation [Bellows and Aubin, 1989; Malaval et al., 1999]. We have previously shown that either chronic or acute (differentiation stage that specific pulse) LIF treatment inhibits bone nodule formation in the RC model, acting on late osteoprogenitors and/or early osteoblasts, and that this effect is antagonized by dexamethasone (Dex) [Malaval et al., 1995; Malaval et al., 1998b]. This action is associated with inhibition of alkaline phosphatase (ALP) and osteocalcin, and stimulation of osteopontin expression, suggestive of an overall inhibition of

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Abbreviations used: LIF: leukemia inhibitory factor; RBM: rat bone marrow; RC: rat calvaria; Dex: dexamethasone; α-MEM: alpha-minimal essential medium; FCS: fetal calf serum; PBS: phosphate buffer saline.

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the osteoblastic phenotype. In a second osteogenesis model, rat bone marrow (RBM) stromal cell cultures, very few bone nodules form in the absence of Dex [Malaval et al., 1994; Herbertson and Aubin, 1997; Maniatopoulos et al., 1988; Aubin, 1999]. One interpretation of the requirement for Dex in RBM cultures is that they comprise mainly early osteoprogenitors, an hypothesis based in part on the fact that Dex appears to induce the differentiation of ALP-negative, presumably less mature progenitors while more mature, ALP-positive progenitors appear to be Dex-independent for differentiation in RC cultures [Turksen and Aubin, 1991]. However, cell sorting experiments on RBM cells suggest a more complex situation, in which both ALP-positive and -negative osteoprogenitors may be Dex-dependant [Herbertson and Aubin, 1997]. Alternatively, the complex cellular milieu and multiple lineages (in particular the high proportion of monocyte–macrophage lineage cells) present in RBM cultures may comprise an environment in which inhibitory factors are downregulated by Dex, e.g. LIF, or other cytokines such as IL-1 [Shadmand and Aubin, 1995 and submitted].

In view of these differences, and because RBM cells contain the progenitors involved in growth plate and trabecular bone formation and remodeling, we have assessed the action of LIF on cell growth and osteogenesis in this model. We have found that response to LIF is biphasic in RBM cultures, with early stage induction and/or acceleration of osteoprogenitor differentiation and late stage inhibition of bone nodule formation.

METHODS

RBM Cell Cultures

RBM cells were harvested and grown as described in [Maniatopoulos et al., 1988]. Briefly, male Wistar rats were killed by cervical dislocation and the femurs were removed in sterile conditions; animal use and care were approved by the institutional Animal Care Committees. After removal of the femoral heads, the marrow was collected by flushing medium through the shafts with a syringe. The marrow cells were precultured for seven days in α-MEM, containing 15% FCS and supplemented with antibiotics, ascorbic acid (28×10^{-5} M), β-glycerophosphate (10 mM) and dexamethasone (10^{-8} M). Secondary cultures were plated at various densities (0.5×10^3 to 4×10^3 cells/cm^2) either in 100 mm or 35 mm dishes, or in 48 well plates, in the same medium.

RC Cell Cultures

RC cells were enzymatically isolated from the calvariae of 21-day old Wistar rat fetuses by sequential digestion with collagenase, as described in [Bellows et al., 1986]; animal use and care were approved by the institutional Animal Care Committees. The populations obtained from each digestion step (I to V) were plated into 75 cm^2 tissue culture flasks in α-MEM, 15% heat-inactivated FCS with antibiotics (50 IU/ml penicillin, 50 IU/ml streptomycin). After 24 h at 37°C, the cells were released with 0.01% trypsin in citrate-saline, and digests II to V were pooled, plated, and grown as described above.

LIF Treatment

In either RBM or RC cultures, the medium was changed 24 h after plating and agonists added if required (Day 1); medium was replaced every second day thereafter. LIF (murine recombinant, 1×10^5U/μg, from Gibco BRL, Gaithersburg, MD) was added in treated dishes at 500 U/ml (5ng/ml; 0.25×10^{-9} M), except in dose-response experiments, either continuously or as "pulses" of various duration during the culture.

RT-PCR Experiments

Total RNA from cells in 4 to 8 100 mm dishes was extracted using the guanidinium thiocyanate procedure [Chomczynski and Sacchi, 1987], at Day 5, 6, 9, 11, and 17 of culture. The mRNAs in 20 μg of total RNA from each sample were reverse transcribed with 10 units of Reverse Transcriptase AMV (Boeringer Mannheim, Mannheim, Germany), using 200 ng of pd(T)$_{12-18}$ (Pharmacia, Uppsala, Sweden) as a template. PCR amplification primers were designed from available sequences of rat LIF (Accession Number: AB010275, start/end of amplified sequence: bp35/370), rat gp130 (M92340, bp1550/2450), rat LIFR (D86345, bp646/1314) and rat osteocalcin (X04141, bp52/345). mRNA for the ribosomal protein L32 (NM_013226, bp6/409) was amplified as an internal control. 40 ng (for osteocalcin and L32) or 400 ng of each sample were amplified with Taq polymerase (Boeringer Mannheim, Mannheim, Germany) using optimized annealing

time and temperature for each set of primers, and aliquots were collected every three cycles during the last 15 amplification cycles. PCR samples were run on 1% agarose gels, visualized by ethidium bromide staining, transfered to nylon membranes (Southern blotting) and detected with specific probes. Probes for gp130, LIF, and LIFR were subcloned from rat PCR products [Malaval et al., 1998b], the rat osteocalcin cDNA was cloned from an ROS 17/2.8 cell cDNA library [Malaval et al., 1994] and the rat L32 cDNA from an osteoblast λgt11 expression library [Malaval et al., 1998b]; in all cases probe identity was confirmed by sequencing of the cDNA insert. Hybridization was assessed by exposure of the Southern blots to X-ray film (Biomax-MS, Kodak, Rochester, NY).

Cell Growth and Bone Nodule Formation Analysis

For cell growth analysis, cultures were arrested at different time points, the cell layers were rinsed in PBS, treated with trypsin and collagenase (1:1 V/V of solutions as above) and harvested cells were counted electronically. For quantitation of bone nodule formation, the dishes were fixed in 3.7% formaldhehyde and stained by the Von Kossa technique, as described [Bellows et al., 1986]. Bone nodules were counted on a grid under low power microscopy.

Data Analysis

Data were subjected to analysis of variance, using the Instat® (version 3.00 for Windows 95, GraphPad Software, San Diego, CA) and Super-ANOVA® (Abacus Concepts, Berkeley, CA) software.

RESULTS

LIF and LIF-Receptor Components are Expressed Throughout Osteogenesis in RBM Cell Cultures

The progression of osteogenesis (i.e. formation and maturation of bone nodules) in RBM cell cultures is marked by a parallel increase in osteocalcin mRNA expression (Fig. 1), from barely detectable during the proliferative phase (Day 5) to the highest levels during bone nodule maturation (Days 9–11, see also Malaval et al., 1994). mRNA for the two chains of the LIF receptor, gp130, and LIFR, were found to be expressed at all time points of the culture, with levels that did not show significant variation

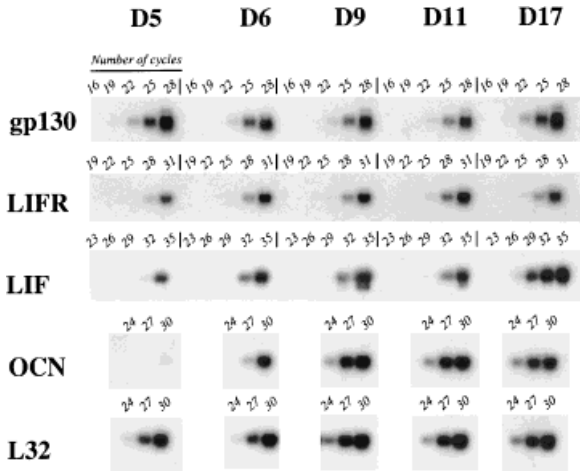

Fig. 1. Time course of expression of gp130, LIF, and LIFR mRNA in osteogenic RBM cell cultures. Total RNA from RBM cells was collected at successive time points and subjected to RT-PCR; the figure shows Southern blot detection of PCR products collected after an increasing number of amplification cycles, as described in Methods. Dx: day of RNA collection; OCN: osteocalcin; L32: ribosomial protein L32, used as internal quantification standard.

with time. LIF mRNA was also expressed throughout the time-course of bone formation, with low levels of expression at early time points and higher expression in late cultures times.

LIF Alters Cell Growth and Saturation Density in RBM Cell Cultures

Continuous treatment with LIF reduced cell numbers during log phase growth in RBM cell cultures. However, a higher cell density/saturation density was attained in LIF-treated compared to untreated cultures (Fig. 2).

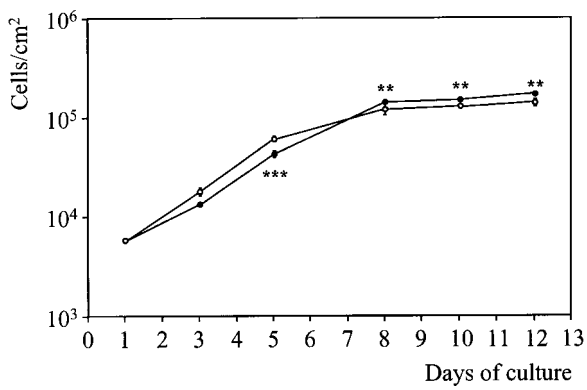

Fig. 2. Growth curve of RBM stromal cell cultures grown with (●) or without (○) 500 U/ml LIF. Results are mean±SD of 4 to 6 dishes; ***: $P < 0.001$, **: $P < 0.01$ vs matched control.

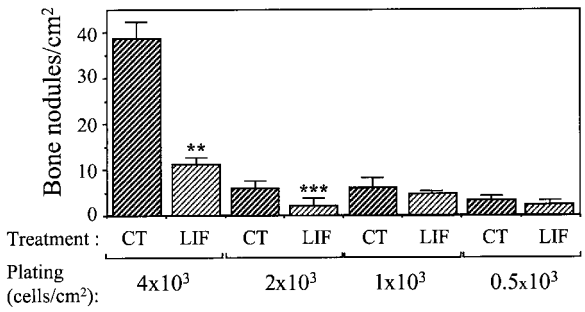

Fig. 3. Cell density dependence of the effects of continuous exposure to LIF on bone nodule formation in RBM stromal cell cultures. Cells were plated at increasing densities and treated continuously with or without 500 U/ml LIF. The cultures were fixed when all nodules were fully mineralized, i.e. at either Day 11 (2000 and 4000 cells/cm^2) or Day 18 (500 and 1000 cells/cm^2). Results are mean±SD of 4 to 6 dishes; ***: $P< 0.001$, **: $P<0.02$ vs matched control.

The Effect of Continuous Treatment With LIF on Bone Nodule Formation is Cell Density Dependent

As documented elsewhere, both the RBM [Malaval et al., 1994] and RC [Bellows et al., 1986] cell systems are dynamic models, in which overt bone nodule formation begins at the end of log growth phase, when cell density reaches a plateau. However, in contrast to what is found by limiting dilution analysis of RC cell cultures [Bellows and Aubin, 1989; Liu et al., 1994; Malaval et al., 1999; Roche et al., 1999], in RBM cell cultures grown with Dex the number of bone colonies formed is not linear with plating density until relatively high cell density is reached, and nodule formation diminishes to zero at low cell density [Herbertson and Aubin, 1997; Aubin, 1999]. Continuous treatment with LIF of RBM cells plated at high cell density reduced the number of bone nodules present at the end of the culture period (Fig. 3), as previously observed in RC cell cultures [Malaval et al., 1995; 1998b]. Interestingly, however, the degree of inhibition was dependent on the plating density, and disappeared at low RBM cell plating density (Fig. 3).

Continuous Treatment With LIF Has Differentiation Stage-Specific Effects on Bone Nodule Formation in RBM Cell Cultures

Analysis of bone nodule formation in RBM cell cultures showed that the action of LIF (500 U/ml; Fig. 4A) on bone nodule formation is biphasic. The first nodules, morphologically

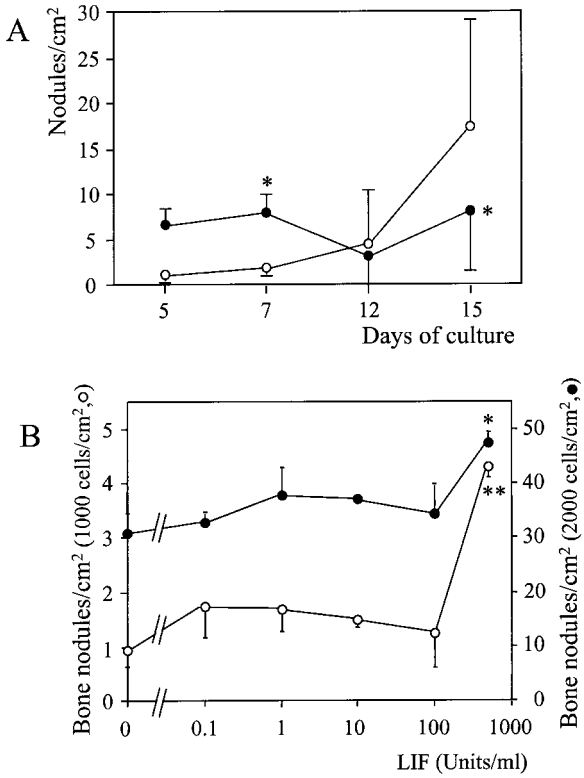

Fig. 4. Biphasic effect of LIF on bone nodule formation in RBM stromal cell cultures. (**A**): Time course of bone nodule formation. Rat bone marrow cells plated at 4000 cells/cm^2 were grown with (●) or without (○) 500 U/ml LIF. Cultures were fixed after 5, 7, 12, or 15 days and processed for nodule counting. Results are mean ± SD of 4 wells; *: $P<0.05$ vs matched controls. (**B**): Dose and density dependence of the effect of LIF on bone nodule formation at early stages of RBM stromal cell cultures. Cells plated at 1000 or 2000/cm^2 were grown for 10 d with various concentrations of LIF and processed for nodule counting. Results are mean ± SD of 4 to 6 dishes; **: $P< 0.01$ vs control.

recognizable as a clump of cuboidal cells surrounded by refractile matrix [Malaval et al., 1999], appeared in high density cultures; the number of such immature nodules were higher at Day 5 and Day 7 in LIF-treated versus untreated/control cultures. On the other hand, when bone nodules were counted later, during the phase of maturation and mineralization, significantly fewer bone colonies were present in LIF-treated cultures, consistent with end-point assessments of LIF effects in continuously treated cells (Fig. 3). When RBM cell cultures plated at two different densities were treated with increasing concentrations of LIF and fixed when early colonies were forming in control/untreated cultures (Day 10), no dose under 500 U/ml had any effect on nodule numbers,

and the stimulatory effect observed was greater in lower density cultures (Fig. 4B).

Pulse Treatment With LIF During Proliferation Stages Increases Bone Nodule Formation in RBM but not RC Cell Cultures

RBM and RC cell cultures were treated with LIF for an increasing number of days starting on Day 1, and the number of bone nodules formed was counted at Day 15, when nodules in untreated control cultures were fully formed and mineralized. LIF treatment of RBM cultures for up to 6 d significantly increased the number of bone nodules in late cultures (Fig. 5A), while longer treatments significantly inhibited bone nodule formation. In contrast, similar early treatments of RC cultures had no effect on the number of nodules formed, while treatment for 6 d or longer reduced nodule formation (Fig. 5B), consistent with our previous observations on RC cells [Malaval et al., 1995].

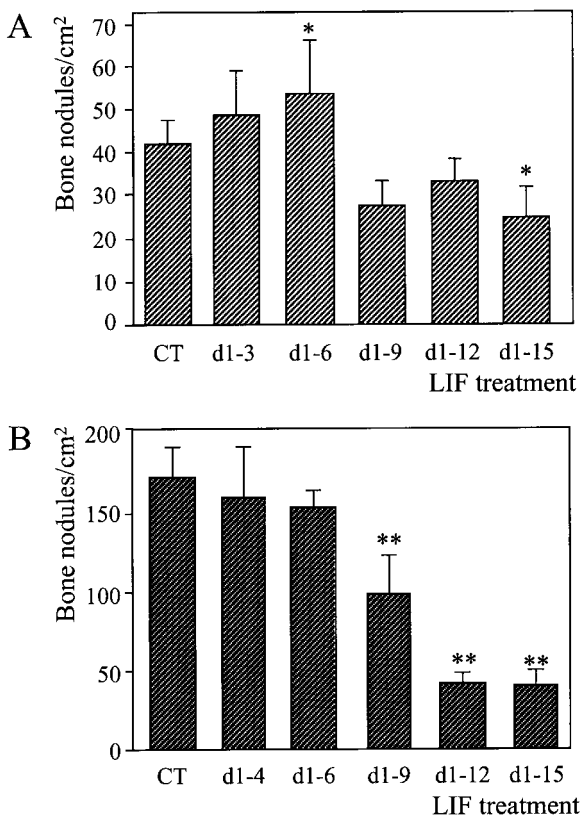

Fig. 5. Effect of the duration of LIF treatment on bone nodule formation. RBM cells plated at 4000/cm^2 **(A)**: or RC cells plated at 2000/cm^2 **(B)**: were treated with LIF (500 U/ml) for various lengths of time starting at Day 1 of the culture; after 15 d, all cultures were fixed and processed for nodule counting. Results are mean ± SD of 4 wells. dx-x: first and last day of LIF treatment. *: $P<0.05$; **: $P<0.01$ vs untreated control.

DISCUSSION

Our previous work on RC cell cultures documented a discrepancy between an inhibitory effect of LIF on osteoblastic differentiation in vitro and a stimulatory effect of this cytokine on bone formation in vivo, the latter observed in transgenic mice [Metcalf and Gearing, 1989] and after local injection [Cornish et al., 1993], consistent with the massive bone loss observed in LIFR knockout mice [Ware et al., 1995]. Cytokines of the LIF/IL6 family have been reported to have anabolic, osteoblast stimulatory actions in bone, and their upregulation due to estrogen loss has been implicated in the increased bone formation that, together with increased bone resorption, follows ovariectomy [Jilka et al., 1998]. However, most studies in vitro have been done on cells already commited to the osteoblast lineage, and addressed either their proliferation or the expression of markers of differentiation such as alkaline phosphatase, thus focusing on the modulation of the osteoblast phenotype, and with contradictory results [Noda et al., 1990; Rodan et al., 1990; Lowe et al., 1991; Ishimi et al., 1992; Bellido et al., 1997]. One study on uncommited stromal cell lines reported a stimulation of the expression of osteoblast markers by these cytokines [Gimble et al., 1994]. In the current study, we assessed the effects of LIF on osteoprogenitor commitment and subsequent differentiation in two different in vitro bone forming models, using their ability to form bone nodules as the endpoint [Malaval et al., 1999], which allows quantification of osteoprogenitor differentiation through a colony assay [Aubin and Herbertson, 1998].

Both components of the LIF receptor complex, gp130 and LIFR, as well as LIF itself, are expressed by cells within the rat bone marrow population (Fig. 1), suggesting that endogenous LIF may have a regulatory action on osteoprogenitor differentiation in RBM cultures, as is the case in the RC model [Malaval et al., 1998b]. Whether osteoblast lineage cells [Martin et al., 1992] or one or several other cell type(s) present in RBM cultures are responsible for biologically significant levels of production of LIF is currently unclear. Previously we found that exogenously-supplied LIF inhibits bone nodule formation in the RC cell culture model when cells are treated continuously from Day 1, or in pulses of several days duration around the time

of confluence or late log phase, suggesting an inhibitory action of LIF on late preosteoblast/early osteoblast stages [Malaval et al., 1995]. We have now found the same effect in RBM cultures, with a similar period of sensitivity (i.e. inhibition after treatment beyond Day 6 of the culture). However, in contrast to what we found in RC cell cultures (Fig. 5B, see also Malaval et al., 1995), we also found that LIF increases the number of nodules present in RBM cell cultures when cells are treated early during the culture time; these nodules are maintained to the endpoint of culture if LIF treatment is restricted to these early stages. Notably, stimulation is significant only at 500 U/ml of LIF, the same concentration required for maximal inhibition of bone nodule formation in cultures grown with Dex and treated continuously with LIF [Malaval et al., 1995]. Early treatments in osteogenic cultures are targeted to proliferation stages rich in precursor/early commited cells. This differentiation-promoting effect of LIF in RBM stromal cultures may be a specific action of LIF on a primitive osteoprogenitor and/or a precursor cell stage more frequent in RBM than in RC cell cultures as judged by their requirement for Dex [Aubin, 1999; Herbertson and Aubin, 1997]. RBM cultures grown without Dex produce very few nodules, and LIF treatment does not replace the requirement for Dex in this model (results not shown). Interestingly, the differentiation-stimulating effects of LIF in pulse treatments early in RBM cultures were modest compared to those seen in the time course experiments. This suggests that the action of LIF may be primarily to accelerate the differentiation of osteoprogenitors, most of which would differentiate into bone nodule-forming osteoblasts later in this model in any case.

The relationship between the number of cells plated and the number of bone nodules formed in RBM cultures is non-linear and falls off to zero at low plating densities, indicating that osteoprogenitor differentiation is cell nonautonomous in this system [Herbertson and Aubin, 1997; Aubin, 1999]. Extrapolation of the limiting dilution curves suggests that at least two different cell types may be involved in osteoprogenitor differentiation in stromal cultures [Aubin, 1999]. When the non-adherent cell fraction of the marrow, or conditioned medium from these cells, is added to RBM stromal cell cultures, the limiting dilution curve becomes linear, suggesting that the cooperating cell type(s) belong to this fraction [Aubin, 1999]. Similarly, the cell plating density-dependence of both the stimulatory and inhibitory actions of LIF on precursor differentiation in the RBM model suggests that one or several other cell types present in the cultures may mediate, or at least modulate, the LIF effects, for instance through the production of one or several other factors. Monocyte/macrophage lineage cells, which are abundant in RBM cultures [Herbertson and Aubin, 1995] and have been hypothesized to regulate osteoprogenitor differentiation in control conditions [Aubin, 1999], could be a target for LIF, but numerous other lineages are expressed in this model [Herbertson and Aubin, 1995]. LIF could also exert its effects through changes in the cellular composition of the RBM stroma, as suggested by the small but significant modulation of cell numbers during both log phase growth and saturation density; these changes could affect bone matrix amount and/or composition (see below), which could in turn feedback on osteoprogenitor differentiation. Thus, differences in osteoprogenitor stages (more or less primitive) and/or cellular environment (presence/abundance of particular accessory cells or not) may explain the different effects of LIF in RC and RBM cell cultures. While tissue origin -membranous versus endochondral bone- may also be involved, local administration of LIF has been shown to strongly stimulate bone formation in calvaria [Cornish et al., 1993], consistent with what was observed in long bones of LIF-transgenic mice [Metcalf and Gearing, 1989], suggesting a similar response of osteoprogenitors in vivo.

Overall, our data indicate that LIF exerts either stimulatory or inhibitory effects on osteoprogenitor differentiation in a manner consistent with differentiation stage-specific effects, and apparently in cooperation with cell–cell and cell–environmental factors that remain to be identified. The striking bone-promoting effect of exogenous LIF in vivo may result from the local presence of a favorable cellular environment and/or a restriction of its action to specific (presumably early) osteoprogenitor stages. As discussed previously [Malaval et al., 1995, 1998b], the latter could be achieved through interaction of LIF with the extracellular matrix, a process that seems to be tightly regulated during the expression of this cytokine [Rathjen et al., 1990; Voyle et al.,

1999]. While in vivo studies are mandatory to clarify these points, experiments on in vitro osteogenic models may provide clues on the factors and mechanisms involved in the regulation of LIF secretion pathways, and in the mediation of its activity during osteoblast differentiation and bone matrix deposition.

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