Interleukin-10 Inhibits the Osteogenic Activity of Mouse Bone Marrow

By Peter Van Vlasselaer, Brigitte Borremans, Rosette Van Den Heuvel, Urbain Van Gorp, and Rene de Waal Malefyt

MURINE bone marrow cells synthesize bone proteins, including alkaline phosphatase (ALP), collagen type I, and osteocalcin, and form a mineralized extracellular matrix when cultured in the presence of β-glycerophosphate and vitamin C. Interleukin-10 (IL-10) suppressed the synthesis of these bone proteins and mineralization without affecting cell proliferation. In addition, mRNA levels for the latter proteins were reduced in IL-10–treated cultures. This inhibitory effect was most outspoken when IL-10 was added before ALP activity peaked, e.g., day 15 of culture. No significant effect was observed when IL-10 was added at later time points. This finding suggests that IL-10 acts at osteogenic differentiation stages that precede ALP expression but is ineffective on cells that progressed beyond this maturation stage. Likewise, IL-10 appeared to be unable to block both ALP activity and collagen synthesis in the preosteoblastic cell lines MN7 and MC3T3 that constitutively synthesize these proteins. Whereas IL-10 did not alter the number of fibroblast colony-forming cells of the marrow, it significantly reduced their osteogenic differentiation potential. In contrast to control cultures, IL-10–treated stroma was unable to either synthesize osteocalcin or to mineralize when subcultured over a 25-day period in the absence of IL-10. The inhibitory activity of IL-10 coincided with significant changes in stroma morphology. Whereas control cultures contained mainly flat adherent polygonal cells, significant numbers of rounded semialcement to nonadherent cells were observed in the presence of IL-10. Scanning and transmission electron microscopy showed that, in contrast to control cultures, IL-10–treated stromas completely lacked a mineralized extracellular matrix. Collectively, these data suggest that IL-10 may have important regulatory effects on bone biology because of its capacity to downregulate early steps of osteogenic differentiation.

© 1993 by The American Society of Hematology.
Fig 1. Effect of IL-10 on the temporal expression of ALP, collagen, osteocalcin, and mineralization in cultures of 5-FU-treated bone marrow. (C) Control cultures; (L) IL-10-treated (2.5×10^3 U/mL) cultures. The results represent the mean ± SD of quadruplicate cultures.

ALP activity. ALP activity was measured as described elsewhere. The cultures were incubated with 0.1 mol/L sodium acetate solution, supplemented with 0.1% Triton X 100 and 5 mmol/L p-nitrophenol phosphate (Sigma 104; Sigma, St Louis, MO), pH 9.6, for 1 hour at 37°C. Absorbance was determined at 405 nm and compared with a p-nitrophenol standard titration curve. ALP activity was expressed as nanomoles of p-nitrophenol formed per minute.

Collagen synthesis. Collagen synthesis was measured as the incorporation of [3H]-proline (Amersham, UK) into collagenase-digestible protein (CDP). Collagenase was purchased from Worthington (UK) and was substantially free of nonspecific protease activity. Cell cultures were exposed to [3H]-proline (1 μCi/well) for 18 hours at 37°C and then washed three times with phosphate-buffered saline (PBS). Collagenase (0.1 mg/mL in PBS) was added for 1 hour and the CDP was measured in a liquid scintillation counter.

Osteocalcin cell enzyme-linked immunosorbent assay (ELISA). The osteocalcin cell ELISA was performed as described elsewhere (Falla et al. submitted for publication). Briefly, the cultures were rinsed with PBS, fixed with 4% formaldehyde for 30 minutes at 4°C and then washed twice with phosphate-buffered saline (PBS). The samples were rinsed with PBS, pH 7.6, and blocked with normal goat serum (1/5 dilution in PBS, pH 7.6; Tago) for 1 hour at 37°C. Rabbit antimouse osteocalcin (kindly provided by Dr R. Bouillon, K. U. Leuven, Leuven, Belgium) was added at a dilution of 1/5,000 and incubated for 2 hours at 4°C. Nonbound serum was removed and the cultures were washed five times with PBS. Horseradish peroxidase-conjugated goat antirabbit Ig serum (1/2,000 dilution in PBS, pH 7.6; Tago) was added for 30 minutes at 4°C. After intensive rinsing, the cultures were incubated in the presence of ABTS substrate solution (ABTS 1 mg/mL + H,O₂ 0.1 μL/mL in citric acid/Na₂HPo₄, 10.5 g/14.2 g/500 mL H₂O) was added until coloration occurred. Absorbance was read in a microtiter reader at 450 nm with a reference of 650 nm. To control nonspecific binding of the antimouse osteocalcin antiserum, nonimmune rabbit serum was used under the same conditions. The sensitivity of this assay was 0.3 ng/mL, whereas no reactivity was observed with FCS.

Calcium determination. Calcium was determined as described elsewhere. The cultures were washed three times with Ca²⁺ and Mg²⁺ free PBS and incubated overnight at room temperature in 0.6 N HCl. The extract was transferred to a 96-well plate and calcium was complexed with o-cresol-phthalein-complexon (Test Combination Calcium; Boehringer Mannheim, Mannheim, Germany). The
colorimetric reaction was read at 570 nm in a spectrophotometer. The absolute calcium concentration was determined in comparison with a standard curve for calcium provided by the vendor.

Quantification of fibroblast colony-forming cells (CFU-F). The number of CFU-F was scored as described elsewhere. Briefly, 5-FU-treated bone marrow cells were prepared as described above and plated in 35-mm culture dishes (Falcon) at $5 \times 10^4$ cells per dish. Replicate cultures were incubated at 37°C for 10 days and fed once by total replacement of the medium. Adherent cells were fixed with methanol and stained with May-Grünwald-Giemsa. Colonies of adherent, fibroblastic cells were scored under a binocular (20 ×).

RNA preparation and Northern blot. Total cellular RNA was prepared by acid guanidine thiocyanate/phenol/chloroform extraction as described elsewhere. The RNA was fractionated by electrophoresis in 1.5% agarose containing 10 mmol/L sodium phosphate after denaturation with glyoxal and dimethylsulfoxide. RNA was transferred to Biodyne filters (Pall) and hybridized to 32P-labeled alkaline phosphatase, collagen type I, and osteocalcin cDNA probes that were kindly provided by Dr B. Mintz, Dr Mon-Li Chu, and the Genetics Institute, respectively. Hybridization and washings were performed according to the manufacturer’s guidelines. Autoradiographic exposure was performed onto Kodak Ortho G X-ray films (Eastman Kodak, Rochester, NY).

Scanning and transmission electron microscopy. For scanning electron microscopy, the cultures were rinsed with PBS and fixed for 1 hour with sodium-cacodylate buffer in 0.1 mol/L phosphate buffer (pH 7.2). After rinsing, the cultures were postfixed for 1 hour with 1% osmium tetroxide in the same buffer. The cultures were subsequently rinsed and progressively dehydrated with alcohol. They were processed for critical point drying (Balzers Union, Liechtenstein) in CO2 and coated with gold (50 nm; Balzers Union). The cultures were observed in a JEOL JSM-F15 microscope. For transmission electron microscopy, the cultures were washed once with PBS and then fixed for 2 hours in a glutaraldehyde (3%), paraformaldehyde (1%), sucrose (5%) solution in sodium-cacodylate buffer (0.1 mol/L, pH 7.2) at 4°C. The cultures were subsequently washed twice in the same buffer and then postfixed for 1 hour in osmium tetroxide in the same buffer. After dehydration of the cultures with alcohol, the samples were embedded in Epon. Sections were prepared on an LKB ultramicrotome, stained with uranyl acetate and lead citrate, and analyzed on a JEOL 1200 EX microscope.

RESULTS

**IL-10 inhibits the osteogenic activity of murine bone marrow without affecting cell proliferation.** Bone marrow cells synthesized several bone proteins, including ALP, collagen type I, and osteocalcin, and formed a mineralized extracellular matrix when cultured in the presence of β-glycerophosphate and vitamin C. To determine the effect of IL-10 on these phenomena, IL-10 was added at the onset of these cultures and bone protein synthesis and the degree of mineralization was determined at 3-day intervals. Figure 1 shows that ALP activity, collagen and osteocalcin synthesis, and calcium incorporation in the extracellular matrix was maximal around days 15, 18, 24, and 27 of culture, respectively. Whereas IL-10 did not affect the ongoing synthesis of ALP and collagen during the first 9 days of the culture, it reduced the levels of both proteins by 40% at the moment of their maximal synthesis. The strongest suppressive effect of IL-10 was observed on osteocalcin synthesis and mineralization, which was reduced up to 80%. This inhibition occurred at the transcription level because Northern blot analysis illustrated that IL-10 reduced the bone protein mRNA level as well (Fig 2). It is unlikely that IL-10 exerts its suppressive effect via specific cytotoxic activity on osteogenic cells because IL-10-treated bone marrow cells synthesized ALP, collagen, and osteocalcin in quantities similar to untreated marrow when IL-10 was washed out of the cell suspension after 12 hours of incubation (data not shown). The inhibitory effect of IL-10 was reversed in the presence of the neutralizing anti–IL-10 monoclonal antibody (MoAb) SXCl, showing the specificity of the reaction (Fig 3). Interestingly, the SXCl MoAb did not alter ALP activity, collagen and osteocalcin synthesis, and mineralization in control cultures, suggesting that IL-10 is not endogenously produced. We next determined whether the suppressive effects of IL-10 were due to a direct inhibiting effect on cell proliferation. To this end, cultures were performed in the presence of IL-10 (2.5 × 10^4 U/mL) and proliferation was determined at 3-day intervals by measurement of [3H]-thymidine incorporation. Figure 4 shows that the level and kinetics of proliferative responses were not affected and even slightly elevated in cultures containing IL-10.

**Differential effect of IL-10 on early and late stages of in vitro osteogenic differentiation.** The temporal expression of bone-related proteins in these cultures reflects the progression of uncommitted cells through the consecutive steps of osteoblastic differentiation, eg, proliferation, matrix maturation, and mineralization. Consequently, this culture system
allowed the determination of the susceptibility of each of these steps to IL-10 activity. Cultures were supplemented with IL-10 (2.5 × 10^3 U/mL) at 5-day intervals and ALP activity, collagen and osteocalcin synthesis, and the degree of mineralization were determined after 30 days of cultivation. Figure 5 shows that bone protein synthesis and mineralization were significantly suppressed when IL-10 was added during the first 15 days of cultivation, but that little or no effect was observed when it was added at later time points. Hence, it appears that the suppressive activity of IL-10 is restricted to osteogenic differentiation stages that precede ALP expression and that cells that were already synthesizing ALP are resistant to the suppressive effects of IL-10. Consistent with this idea was the finding that IL-10 (5 × 10^3 U/mL) did not affect ALP activity and collagen synthesis by the preosteoblastic cell lines, MN733 and MC3T3, which synthesize ALP and collagen constitutively (Fig 6).

IL-10 does not affect the frequency of CFU-F, but changes their osteogenic differentiation potential. The above findings suggest that IL-10 exerts its suppressive activity on early, but not on late steps in osteoblastic differentiation. Because the fibroblastic compartment of the bone marrow is believed to harbor precursor cells of the osteogenic lineage, IL-10 activity could a priori result from a suppressive effect on the number of CFU-F. To analyze this possibility, CFU-F colony formation was measured in the presence of...
different concentrations of IL-10. Figure 7 shows that, although concentrations were used that completely blocked the formation of a mineralized matrix, IL-10 did not exert a notable effect on the number or size (data not shown) of the CFU-F colonies measured after 10 days of culture. This illustrates once more that IL-10 does not act via a specific cytotoxic effect on osteogenic cells. Indeed, because the latter cells represent 30% of the CFU-F (Falla et al, submitted for publication), the depletion of this population would be noticed in CFU-F analysis. In addition, the effect of IL-10 on the osteogenic potential of the CFU-F was determined by growing the cells in the absence or presence of IL-10 (2.5 × 10^3 U/mL) until mineralization occurred in the control cultures, eg, day 15. At that time point the cells were subcultured for 30 additional days in the absence of IL-10. At 5-day intervals, ALP activity, collagen and osteocalcin synthesis, and the degree of mineralization were determined. Figure 8 shows that ALP activity and collagen synthesis were comparable in control and IL-10-treated cultures. In contrast, osteocalcin synthesis and mineralization increased in function of time in the control cultures, whereas these parameters did not exceed background levels in IL-10–pretreated cultures.

**Morphologic changes in bone marrow cultures induced by IL-10.** Coinciding with the suppressive activity on bone protein synthesis and mineralization, microscopic observation showed dramatic changes in the morphology of IL-10–treated bone marrow cultures (Fig 9). Whereas control cultures were predominantly composed of adherent, flat polygonal cells, large numbers of rounded, semiadherent, and nonadherent cells appeared in the cultures containing IL-10. The latter cells formed clusters in close contact with

![Graphs showing ALP activity, Collagen, Osteocalcin, and Calcium](image-url)
Fig 7. Effect of different concentrations of IL-10 on the frequency of CFU-F in 5-FU-treated bone marrow. IL-10 was added from the start of the culture. Ten days later, the fibroblastic colonies were stained with May-Grünwald-Giemsa and counted. The results represent the mean ± SD of quadruplicate cultures. Control cultures in the absence of IL-10 gave 540 ± 70 CFU-F colonies.

Fig 8. Long-term effect of IL-10 on ALP activity, collagen and osteocalcin synthesis, and mineralization in cultures of 5-FU-treated bone marrow. Marrow cells were cultured in the presence or absence of IL-10 (2.5 X 10^3 U/mL) and were harvested 15 days later using versene. Subsequently, they were subcultured at 10^4 cells per well in 96-well multiwell plates for 25 additional days in the absence of IL-10. ALP activity, collagen and osteocalcin synthesis, and mineralization was scored at 5-day intervals. (○) Control cultures; (●) IL-10-treated cultures. The results represent the mean ± SD of triplicate cultures.

DISCUSSION

Cultured bone marrow from normal and 5-FU-treated animals synthesizes bone-related proteins, including ALP, collagen type I, and osteocalcin, a protein that is exclusively synthesized by cells of the bone lineage. Moreover, a mineralized matrix is formed in these cultures after prolonged culturing. The temporal expression of these different bone proteins and mineralization reflects the progression of osteogenic precursor cells through the stages of osteoblastic differentiation in vitro. IL-10 did not affect ongoing synthesis of ALP and collagen during the first 9 days of the culture, but maximally suppressed these proteins at days 15 and 18, respec-
Fig 9. Effect of IL-10 on the morphology of bone marrow stroma cultures. Light microscopy (phase contrast, × 320) image of 15-day-old control (A) and IL-10–treated (2.5 × 10^3 U/mL) (B) bone marrow cultures. Flat, adherent polygonal cells characterize the control cultures. Clusters of semiadherent and nonadherent cells in contact with reticular (black arrow) and fibroblastic (white arrow) cells characterize the IL-10–treated cultures. Scanning electron microscopy of 25-day-old control (C, × 800) and IL-10–treated (2.5 × 10^3 U/mL) (D, × 700) cultures. Note the mineralized collagenous matrix (white arrow) surrounding the fibroblastic cells (black arrow) in the control culture. IL-10–treated cultures showed no mineralized matrix and were composed of monocytic cells (white arrow) showing membrane ruffling in close contact with flattened fibroblast-like cells (black arrow). Transmission electron microscopy of 15-day-old control (E, × 2,500) and IL-10–treated (2.5 × 10^3 U/mL) (F, × 1,800) cultures. Control cultures showed fibroblast-like cells with a large nucleus and an outspoken RER embedded in a mineralized collagenous matrix (black arrow). Sites of initial mineralization are shown by the white arrows. Cultures in the presence of IL-10 showed no mineralized matrix and were composed of rounded cells displaying large vacuoles that occasionally contained phagocytized material (black arrow head).
tively. These suppressive effects are not due to a shift in kinetics because identical expression patterns of bone-related proteins and mineralization were observed in the absence or presence of IL-10. The inhibitory effects are specific for IL-10 because they were completely neutralized in the presence of the IL-10-specific MoAb SXC 1. Interestingly, this MoAb did not affect bone protein synthesis and mineralization in control cultures, indicating that IL-10 was not endogenously produced. IL-10 activity on bone protein synthesis and mineralization does not appear to result from an overall suppressive effect on proliferation, as measured by thymidine incorporation. However, care should be taken because it has been shown that IL-10 has differential effects on different cell types \(1^{6-10,43,44}\) and may therefore stimulate proliferation of a particular cell population while blocking DNA synthesis of osteoprogenitor cells. However, it is unlikely that IL-10 specifically suppressed the proliferation of osteoprogenitor cells because IL-10 did not affect the number or size (data not shown) of the CFU-F colonies that are believed to harbor the osteoprogenitor cells.\(^{33}\) In addition, IL-10 did not affect DNA synthesis in the preosteoblast cell lines MN7 and MC3T3 (data not shown).

IL-10 affected cellular events that determine osteogenic differentiation and that precede the acquisition of ALP activity, collagen and osteocalcin synthesis, and the potential of marrow cells to form a mineralized extracellular matrix. Indeed, suppressive effects on bone protein synthesis and mineralization were only observed when IL-10 was added to the cultures before maximal ALP synthesis occurred, eg, at day 15 of culture. IL-10 was ineffective when added after this time point. This finding suggests that IL-10 is able to block de novo but not ongoing synthesis of bone proteins. This notion was supported by the observation that IL-10 was ineffective in reducing ALP and collagen synthesis in the preosteoblastic cell lines MN7 and MC3T3, which constitutively synthesize these proteins. Although these data roughly indicate at which level IL-10 affects osteogenic differentiation, its mechanism of action remains unsolved. Furthermore, it remains to be determined whether its effect is direct or indirectly mediated by other factors. In this context, recent experiments showed that transforming growth factor-\(\beta\) (TGF-\(\beta\)) synthesis is significantly reduced in IL-10-treated bone marrow cultures (Van Vlasselaer et al, manuscript submitted). This is of particular interest because the role of TGF-\(\beta\) in osteoblastic differentiation and proliferation has been documented before.\(^{42,43}\)

Osteogenic precursor cells are believed to be undifferentiated mesenchymal cells belonging to the fibroblastic compartment of the bone marrow.\(^{45,46}\) It is obvious that changes in the frequency or the outgrowth of CFU-F in vitro could a priori be a valid explanation for the suppressive activity of IL-10. However, this study showed that, despite the fact that IL-10 was used at concentrations that completely blocked the formation of mineralized nodules, it did not affect the number or size of CFU-F colonies. Consequently, we suggest that IL-10 directs CFU-F differentiation away from bone development. Furthermore, the observation that IL-10-treated cultures were unable to synthesize osteocalcin and to mineralize upon subcultivation in the absence of IL-10 supports the notion that these effects of IL-10 on bone marrow stroma differentiation are irreversible under the culture conditions used.

The results shown in this report could hypothetically be explained by a selective toxicity of IL-10 to cells capable of producing ALP, collagen, and osteocalcin. It is at least clear that IL-10 is not cytotoxic for cells committed to the osteogenic lineage because IL-10-induced suppression occurred only when IL-10 was added to the culture before ALP activity started to increase. Furthermore, it is unlikely that IL-10 exerts cytotoxic effects on earlier osteogenic differentiation stages because (1) IL-10-treated bone marrow showed osteogenic activity equal to control cells when IL-10 was washed out of the cell suspension after 12 hours of incubation; (2) IL-10 does not reduce CFU-F frequencies, although this could be expected based on the fact that osteogenic cells represent approximately 30% of the CFU-F population; and (3) recent data showed that the suppressive effect of IL-10 could be reversed by adding exogenous TGF-\(\beta\)1 to the culture (Van Vlasselaer et al, submitted for publication).

Compared with control cultures, which are composed of predominantly flat polygonal cells, stromal morphology was drastically changed in the presence of IL-10. In the latter cultures semiadherent and nonadherent cells occurred as “cobblestone-like” structures in close contact with underlying stroma of reticular and fibroblastic cells displaying extended cytoplasmic protrusions. In many respects, the IL-10-containing cultures resembled hematopoietic cultures of the Dexter type.\(^{43}\) This is again compatible with the induction of hematopoiesis observed in cultures supplemented with IL-10 and further supports our hypothesis that IL-10 directs stromal differentiation towards hematopoietic support. Scanning electron microscopy showed the presence of a mineralized matrix that fills the extracellular spaces in control but not in IL-10–treated cultures. Moreover, the latter cultures contained large flat cells that formed a “blanket cell” stroma characteristic for cultures in which active hematopoiesis occurs.\(^{43}\) In transmission electron microscopy, control cultures were mainly composed of fibroblastic cells with a well-developed rough endoplasmatic reticulum and a large nucleus. Collagenous material with electron dense matrix vesicles could be observed between these cells. In contrast, the IL-10–treated cultures contained predominantly monocytic cells displaying membrane ruffling and phagocytized material within their vacuoles.

Collectively, our data show the suppressive activity of IL-10 on the in vitro osteogenic activity of mouse bone marrow. This novel biologic activity does not result from a direct effect of IL-10 on the expression of bone related proteins but results from IL-10 inhibitory effect on the commitment of mesenchymal cells towards the bone lineage. These findings may be of particular importance in certain in vivo conditions in which IL-10 synthesis is increased. In this context, the role of IL-10 in bone pathologies associated with (1) Th 2-mediated infectious disorders, eg, lepromatous Mycobacterium leprae\(^{46}\); or (2) excess numbers of mast cells, eg, systemic mastocytosis\(^{47}\); awaits further investigation.
ACKNOWLEDGMENT

The authors thank F. Vander Plaetse and Lilly Diercks for technical and administrative help.

REFERENCES

1. Gowen M: Cytokines and Bone Metabolism. Boca Raton, FL, CRC, 1992

2369


47. Graves LL, Stechschulte DJ, Morris DC, Lukert BP: Inhibition of mediator release in systemic mastocytosis is associated with reversal bone changes. J Bone Miner Res 5:1113, 1990
Interleukin-10 inhibits the osteogenic activity of mouse bone marrow

P Van Vlasselaer, B Borremans, R Van Den Heuvel, U Van Gorp and R de Waal Malefyt