Interleukin-11 Inhibits Bone Formation In Vitro

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Summary.
The effects of interleukin-11(IL-11) on the differentiation of osteoblast precursors was tested using a bone nodule forming assay in rat calvaria cell cultures. IL-11 caused a dose dependent inhibition of nodule formation, with 500U/ml IL-11 resulting in complete inhibition of nodule formation. IL-11 also caused a reduction in alkaline phosphatase expression in these cultures. These effects are similar to, but more potent than, the actions of IL-6 on these cells. These results indicate that IL-11 is an osteotropik cytokine and suggest that IL-11 may be an important inhibitor of bone formation in health and disease.

Introduction.
Interleukin-11 (IL-11) is a recently described cytokine which was originally isolated from an immortalized bone marrow stromal cell line (1). It is known to have a number of activities including stimulation of various stages of haemopoiesis and lymphopoiesis, for example in promoting growth of plasmacytoma cells and growth of early haematopoietic progenitor cells (1, 2), stimulation of acute phase protein secretion by hepatocytes (3), and inhibition of adipocyte formation by pre-adipocytes (4). Many of these reported activities are similar to those of IL-6, although IL-11 is biochemically quite distinct from IL-6. There is increasing evidence that IL-6 may play a role in the regulation of bone metabolism. IL-6 is synthesised by osteoblasts, stimulates osteoclast formation, and may inhibit osteoblast metabolism (for recent review see (5)). In view of the findings that IL-11 has many similar properties to IL-6, is produced by bone marrow stromal cells, and inhibits adipocyte differentiation, we have investigated the possibility that IL-11 may inhibit bone formation by osteoblasts in vitro using a bone nodule-forming assay system in neonatal rat calvaria (RC) cell cultures.

Materials and Methods.
Bone nodule assays were carried out using the methods described by Bellows and co-workers (6). Briefly, calvariae were removed from neo-natal Wistar rats and following removal of adherent soft tissue were sequentially digested in collagenase. The reaction was stopped after 10, 20, 40, 60 and 80 minutes and the cells released at each time point were collected. Cell populations released after the first two digestions were discarded, and the third to the fifth populations, which are enriched with cells of the osteoblast lineage (6, 7), were pooled and plated in T-75 culture flasks for 24 hours in αMEM (Gibco, Paisley, Scotland) supplemented with 15% foetal bovine serum (ICN Flow, Irvine, Scotland), 50U/ml penicillin G, 50µg/ml streptomycin, 0.3µg/ml amphotericin, 10mM sodium β-glycerophosphate, and 50µg/ml ascorbic acid (all obtained from Sigma Chemical Company, Poole, Dorset, England). All reagents used throughout experiments were of tissue culture grade and had reported endotoxin concentrations of < 10EU/ml. Cultures were kept at 37°C in a humidified atmosphere with 5% CO₂. After 24 hours cells were released by trypsin/EDTA treatment (Sigma) and replated into 30mm tissue culture dishes at a density of 6 x 10³ cells/cm².

After a further 24 hours cultures were re-fed with medium plus recombinant human IL-11 at concentrations ranging from 0 - 500 U/ml (specific activity 2.5 x 10⁶ U/mg). IL-11 was a generous gift of the Genetics Institute, Cambridge MA USA. Cultures were exposed to IL-11 for 3 days, following which they were re-fed three times each week with medium without IL-11 for up to 21 days. In further experiments cultures were fed with 10U/ml IL-11 for periods ranging from 0 - 7 days. Cultures were then fixed with 10% formal saline and stained with Alizarin Red to demonstrate mineralised bone nodules (8). Total numbers of mineralised bone nodules in dishes after 21 days in culture were counted by computer-assisted image analysis (Seescan, Cambridge, UK).

In order to measure alkaline phosphatase activity, RC cells were plated into 24-well culture dishes 24 hours following isolation at a density of 6 x 10³ cells/cm² as before. Cultures were exposed to IL-11 for 3 days following which cells were fixed in cold methanol and suspended in 0.05M tris buffer pH 7.4 with 0.1% Nonidet NP40 (Sigma). After sonication, supernatants were assayed colorimetrically for alkaline phosphatase activity by measuring release of p-nitrophenol from p-nitrophenyl phosphate spectrophotometrically at 405nm. Cell proliferation was assessed by measuring release of [³H]-thymidine into the supernatant of cultures following exposure to 100U/ml IL-11 for 24 h by pulse labelling for 2 hours with bromodeoxyuridine (BrDU). Cells were fixed in ethanol and labelled cells were demonstrated immunocytochemically using anti-BrDU antiserum (Dako) and Avidin Biotin Complex system.

Results.
Bone nodules similar to those described by Bellows and co-workers (6) were seen to begin formation in control cultures after 10 days. In cultures treated with 50U/ml IL-11 or above, nodule formation was not seen before day 13. Total numbers of bone nodules were determined after 21 days in culture. Results were expressed as the percentage of nodules compared with control cultures. Mean number of nodules in control cultures varied between 154 ± 7.9 and 232 ± 16.4 in different experiments. IL-11 caused a dose-dependent reduction in bone nodule
formation, with complete inhibition seen following treatment with 500 U/ml (Figure 1). There was no difference in the mean area of individual bone nodules between test and control groups (eg. 1.83 ± 0.11 mm² in control cultures; 1.73 ± 0.22 mm² in 100U/ml IL-11 cultures; P > 0.1, mean ± sd).

Inhibition of bone nodule formation was seen following treatment with 10U/ml IL-11 during the first day of exposure; no greater inhibition was seen following longer periods of exposure to IL-11 (Figure 2).

In addition to effects on nodule formation, IL-11 also resulted in a dose-dependent reduction in alkaline phosphatase activity in 4-day old RC cultures (Figure 3). There was no significant difference in cell numbers after 4 days in culture between test and control groups (eg. 1.22 ± 0.03 x 10⁵ cells in control cultures; 1.41 ± 0.13 x 10⁵ cells in cultures with 100U/ml IL-11, P > 0.1). The proportion of mid-log phase cells synthesising DNA, as measured by BrdU labelling, was not affected by 24 hours exposure to 100U/ml IL-11 : Control cultures 32.2±4.5 % labelled; Test Cultures 35.2 ± 0.6 % labelled, P > 0.7.

Discussion.

The bone nodule assay used in these studies has been extensively characterised (6,9, 10), and those studies have suggested that the assay acts akin to a colony forming assay of osteoblast differentiation. In the current experiments the reduced numbers of bone nodules seen in test cultures after 21 days suggests that IL-11 inhibits differentiation of osteoblasts when osteoprogenitor cells are exposed to IL-11 at early times in culture. This is further supported by the findings of reduced alkaline phosphatase activity in 4 day cultures treated with IL-11. IL-11 did not affect total cell numbers in these cultures or BrdU labelling index, which suggests that it does not act specifically on osteoprogenitor cell proliferation, although this issue requires further study.

Interleukin-11 was originally isolated from a bone marrow stromal cell line (1) and has also been found in fibroblast lines (11). The results of the study reported here suggest that IL-11 may act as a paracrine inhibitor of bone formation. It is not clear if such a mechanism might be confined to the bone marrow spaces and endosteal surfaces of bone. Information is required on the cells which produce IL-11 and factors which might regulate its production before the possible role of IL-11 on osteoblast function can be fully evaluated.

There is increasing evidence that IL-6 may inhibit bone formation in addition to its effects on osteoclast formation (5). We have carried out similar bone nodule assays to those described here using IL-6, and have shown that IL-6 inhibits bone nodule formation in RC cell cultures in the same way that we report here using IL-11 (12). However, IL-11 is a considerably more potent inhibitor of bone nodule formation than IL-6, with approximately 50%M IL-6 reducing nodules by ≈ 50%, whereas in the experiments described here 2nM IL-11 (= 100 U/ml) inhibited nodule formation by approximately 80%

In addition to IL-11, other factors which share many similar functional activities with IL-6, but which are distinct biochemically, include leukaemia inhibitory factor (LIF) (13), and oncostatin M (14). In contrast to IL-6 and IL-11, there is evidence that LIF may stimulate bone formation (15). Little is currently known about the effects of oncostatin M on bone metabolism.

In addition to its potent inhibitory effects on osteoblast metabolism shown in the study reported here, a recent report has suggested that IL-11 may also promote osteoclast formation and stimulate bone resorption (16). This report leads further support to our data suggesting that IL-11 may be a significant osteotropic cytokine. However further studies are required in order to investigate the true physiological and pathophysiological significance of the inhibition by IL-11 of bone formation.

References.