Inflammatory cytokines regulate proliferation of cultured human osteoblasts

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We investigated the effects of various pro-inflammatory cytokines on the proliferation rate of isolated human osteoblastic cells in primary cultures.

Interleukin-1ß (IL-1ß) and tumor necrosis factor-ß (TNF-ß) time- and dose-dependently enhanced the proliferation of human osteoblasts. Both of these cytokines also enhanced endogenous prostaglandin E_2 (PGE_2) formation. Exogenous PGE_2 dose- and time-dependently stimulated cell proliferation. However, the stimulatory effects of IL-1ß and TNF-ß on osteoblast proliferation were not abolished by indomethacin, indicating a direct effect by these cytokines on the rate of proliferation. TNF-α stimulated proliferation at low doses, while it significantly inhibited proliferation at higher concentrations (at and above 100 pM) and with prolonged incubation times. This biphasic effect was unaffected by indomethacin. Interleukin-6, finally, did not affect the rate of proliferation.

Our findings show that inflammatory cytokines may stimulate or inhibit the proliferation of isolated human osteoblasts, depending on concentration and time.

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Inflammatory cytokines have pronounced paracrine and autocrine effects on bone cells. Interleukin 1 (IL-1), cachexin (TNF-α), lymphotoxin (TNF-ß) and interleukin 6 (IL-6) have attracted most of the interest. IL-1 and TNF-ß are both potent inducers of bone resorption. They are produced by cells in the inflammatory infiltrate and together constitute the activity called osteoclast activating factor, OAF (Stashenko et al. 1989). TNF-α, produced by inflammatory cells, can stimulate osteoclastic activity (Mundy 1993). IL-6, finally, has been implicated as a mediator of the enhanced osteoclastic activity due to estrogen loss (Jilka et al. 1992). These cytokines also have pronounced effects on osteoblastic activity; IL-1 and TNF-α inhibit formation of specific proteins in osteoblasts (Stashenko et al. 1989, Evans et al. 1990b, Taichman and Hauschka 1992), e.g., osteocalcin and alkaline phosphatase, and have therefore been suggested to cause the inhibition of osteoblastic activity seen in myeloma. These two cytokines also enhance osteoblastic proliferation and could therefore be involved in the coupling process seen in normal bone turnover, in callus formation or in ectopic bone formation (Evans et al. 1990a, Bodo et al. 1992, Rickard et al. 1993). The effect of IL-6 on osteoblastic proliferation is less clear. Mitogenic effects of IL-6 on rat osteosarcoma cells have been reported (Fang and Hahn 1991), but this has not been seen in isolated human osteoblasts (Littlewood et al. 1991). Another important aspect of these cytokines is their ability to induce prostaglandin formation in osteoblasts (Ellies and Aubin 1990, Littlewood et al. 1991). It has been shown that the inhibitory effects on alkaline phosphatase and osteocalcin synthesis in osteoblasts are partly mediated via PGE_2 (Evans et al. 1990b, Zheng et al. 1992). It is not clear to what extent the prostaglandins play a role in the effects of these cytokines on osteoblastic proliferation. It has been reported that IL-1 induces cell proliferation only if the endogenous prostaglandin formation is abolished.

We used a new fluorometric proliferation assay, based on the fluorescent probe Alamar Blue, to investigate the effects of the cytokines IL-1ß, IL-6, TNF-α, and TNF-ß on the proliferation rate of primary cultures of human osteoblastic cells (hOBs). Special attention was given to the possible involvement of prostaglandin formation in the proliferative response to these cytokines.

Material and methods

Material

Alpha modification of Eagle's medium (α-MEM), Dulbecco's modification of Eagle's medium (D-MEM), penicillin, streptomycin, L-glutamine,

trypsin-EDTA, and fetal calf serum (FCS) were purchased from Gibco, Life Technologies Ltd., Paisley, Scotland, UK; PGE_2, IL-1ß, IL-6, TNF-α, TNF-ß and collagenase from Sigma Chemical Co., St. Louis, MO, USA; PGE_2 radioimmunoassay kit from DuPont Company, Wilmington, DE, USA. Insulin-like growth factor-I (IGF-I) was kindly provided by Kabi Pharmacia AB, Stockholm, Sweden. The Alamar Blue growth indicator is commercially available at AccuMed, Westlake, OH., USA. The 96-well scanning fluorometer (Fluoroscan II) was obtained from Labsystem, Stockholm, Sweden.

Cell culture

Primary isolations of human osteoblastic cells were obtained from cultured bone fragments taken from patients during a bone grafting procedure in spinal surgery. The cells were isolated as originally described by Beresford et al. (1984). Briefly, trabecular bone from the iliac crest was cut into pieces (2–3 mm), thoroughly rinsed with PBS, incubated for 2 hours in 37 °C with collagenase 1 mg/mL and then cultured in α-MEM supplemented with 10% FCS, 60 mg/mL of penicillin, 50 mg/mL of streptomycin and 2 mM L-glutamine. After 35–50 days, the culture dishes were confluent with cells that had migrated from the explants and subsequently proliferated. These cells express many aspects of the osteoblastic phenotype, including cyclic AMP response to PTH, osteocalcin synthesis, alkaline phosphatase activity and type I collagen synthesis (Beresford et al. 1984, 1986, Jonsson et al. 1993). The cells were detached, using trypsin-EDTA and plated at a density of 2000 cells/well in 96-well flat-bottomed Nunclon microwell plates (Nunc, Roskilde, Denmark) in which the experiments were performed. Only first-passage cells were used in the experiments. Cells from 15 donors were used in the experiments. We detected no difference between the various donors, regarding proliferation capacity in the isolated cells.

Measurement of proliferation

Osteoblastic cells were plated at a concentration of 2000 cells/well in α-MEM containing 10% FCS and antibiotics. They were allowed to adhere for 24 hrs. Thereafter a medium containing the experimental agents and 5% FCS was added and the plates were incubated for different periods of time. Half of the media were replenished every fourth day. At the end of the experiments, the media were removed and the cells were rinsed with PBS before D-MEM, without phenol red or FCS, containing 10% Alamar Blue (v/v), was added. The wells were incubated with Alamar Blue for 5 hrs before measurements. Thereafter the plates were brought to a fluorometer equipped with a xenon lamp and a broad band interference filter exciting fluorescence at 544 nm. The light emitted from a vertical light path on each well in 96-well microtiter plates was read at 590 nm. The fluorescence thus obtained is directly proportional to the cell number in each well (Jonsson et al. 1997). The fluorescence obtained in control wells was set to 100% and effects of agonists were compared to this value.

Prostaglandin E_2 formation

Osteoblastic cells were plated at a concentration of 10000 cells/well and cultured as described above. At confluence the cells were challenged with agonists and incubated for 24 hrs. At the end of the incubation period supernatants were collected and stored at –20 °C pending analysis. The amounts of PGE_2 in the samples were analyzed by a radioimmunoassay kit. Intra-assay variability was 6% and inter-assay variability was 21%. Sensitivity as reported by the manufacturer was < 0.2 pmol/mL.

Results

Since prostaglandins are known to mediate some of the effects of IL-1ß, IL-6, TNF-α and -ß, we first evaluated the effect of exogenous PGE_2 on cell proliferation. Consistently, PGE_2 dose- and time-dependently stimulated cell proliferation in hOBs (Figure 1). Among the cytokines investigated, IL-1ß, TNF-α and TNF-ß were potent inducers of PGE_2 formation in hOBs, while IL-6 did not affect the PGE_2 formation (Table 1). IL-1ß, TNF-ß and TNF-α, all stimulated the growth rate of hOBs by a non-prostaglandin-dependent mechanism—i.e., the stimulatory effects were not abolished by addition of indomethacin 1 mM (Figures 2–4). Interleukin-6, on the other hand, did not affect cell growth in hOBs (1-10 pM, 12 days culture; Table 2). Although IL-1ß and TNFs all directly stimulated cell growth, there were important differences in their effects. The stimulatory effect by IL-1ß was seen after 8 days of treatment, while TNF-ß- and TNF-α-induced proliferation were detected already after 4 days in culture (Figures 2–4). Indomethacin sensitized osteoblasts to treatment with IL-1ß, with stimulatory effects at 3 pM in the presence of indomethacin 1 mM and at 30 pM in the absence of indomethacin (Figure 2). TNF-ß-induced proliferation was totally unaffected by co-treatment with indomethacin. Finally, TNF-α at 1–10 pM concentration dose- and time-dependently stimulated the rate of proliferation in hOBs. At and above 30 pM, however, TNF-α caused a marked inhibition of cell prolifera-

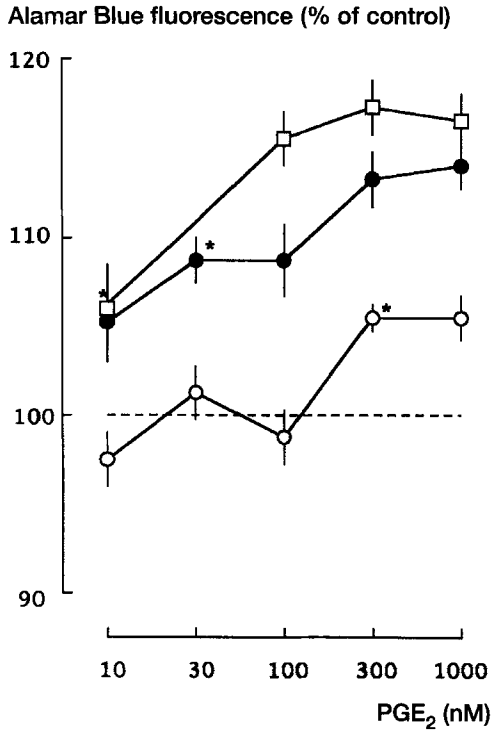

Figure 1. Effect of PGE_2 on osteoblastic proliferation. Confluent primary cultures of human bone-derived cells were trypsinated and 2000 cells/well were seeded in 96-well culture plates. The cells were allowed to adhere overnight before PGE_2 (10–1000 nM) was added. Half of the media were replenished every third day, with media containing agonists, and the experiment was continued for 4, 8 and 12 days. At the end of the experiment, the Alamar Blue assay was performed, as described in methods. The data are mean ± SEM of 16 wells in each group, and presented as percent of control, with control values arbitrarily set to 100%. ○ 4 days in culture, ● 8 days in culture, □ 12 days in culture. * First value significantly different from untreated control, $p<0.05$, by ANOVA

Table 1. Effects of IL-1ß, TNF α, ß and IL-6 on PGE_2 formation in human osteoblasts. Data are mean (SEM) of 8 wells in each group

Agonist	Concentration (pM)	PGE_2 (ng/mL)
–	–	5.9 (1.2)
IL-1ß	10	15 (1.5) [a]
IL-1ß	100	271 (51.0) [a]
TNF-α	10	21 (3.6) [a]
TNF-α	100	36 (2.8) [a]
TNF-ß	10	18 (2.2) [a]
TNF-ß	100	27 (3.0) [a]
IL-6	10	5.8 (1.3)
IL-6	100	5.6 (1.0)

Confluent primary cultures of human bone-derived cells were trypsinated and 10,000 cells/well were seeded in 96-well culture plates. The cells were allowed to adhere overnight before the agonists were added to the culture media without FCS. After 48 hrs, the media were harvested and the amount of PGE_2 released analyzed by radioimmunoassay.

[a] Significantly different from untreated control, $p<0.05$, by ANOVA.

Table 2. Effect of IL-6 on osteoblastic proliferation. Data are mean (SEM) of 16 wells in each group and presented as percent of control with control values arbitrarily set to 100%

Agonist	Concentration	AB-Fluorescence (% of control)
–	–	100 (1.1)
IGF-I	100 nM	114 (1.0) [a]
IL-6	1 pM	102 (0.8)
IL-6	3 pM	101 (1.0)
IL-6	10 pM	100 (0.8)

For method, see Figure 1.

tion. Thus, there were significantly fewer osteoblasts in cultures treated with 100 pM TNF-α for 12 days than in controls. This biphasic effect by TNF-α on osteoblastic cell growth was also unaffected by co-treatment with indomethacin 1 mM (Figure 4).

Discussion

During fracture healing, inflammatory cells are present in the fracture area (Andrew et al. 1994). In ectopic bone formation there is also an inflammatory phase preceding bone formation. Thus, presence of inflammatory cells appears to be a general feature in bone formation. There may also exist autocrine actions of cytokines on osteoblastic cells, since both IL-1 and TNF-α are produced by osteoblasts (Gowen et al. 1990, Keeting et al. 1991). Both these cytokines also enhance the formation of IL-6, PGE_2, and IGF-I in osteoblasts, factors that have been implicated in the regulation of osteoblastic activity. This suggests that inflammatory cytokines are involved in the regulation of osteoblastic bone formation, either by para- or autocrine mechanisms (Zheng et al. 1992). The bone-forming process is composed of two distinct phases. First, a population of immature mesenchymal cells becomes committed to the osteoblastic cell lineage and starts to proliferate, and in a second phase they differentiate. During the differentiation process, they gradually mature and secrete the bone-specific proteins that constitute the organic matrix and subsequently cause it to mineralize (Stein and Lian 1993).

It is clear that the proliferative phase is a crucial step which to a large extent determines the amount of

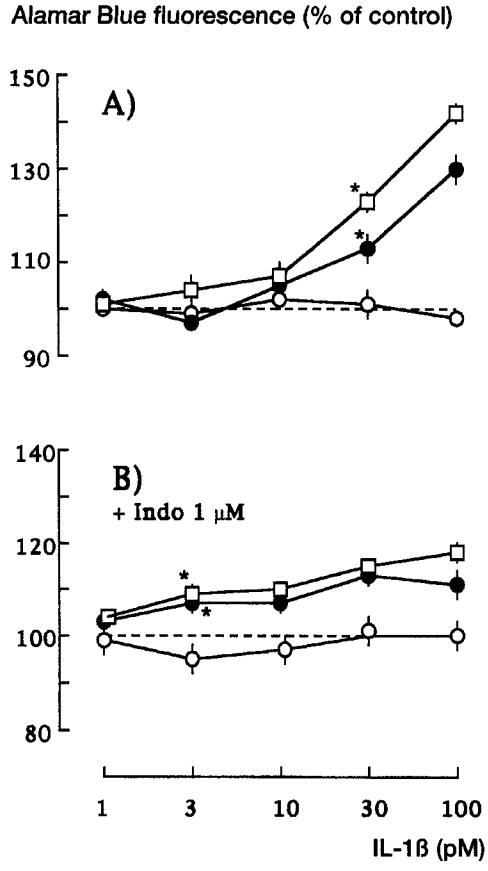

Figure 2. Effect of IL-1ß with (A) or (B) without indomethacin on osteoblastic proliferation. For method, see Figure 1. ○ 4 days in culture, ● 8 days in culture, □ 12 days in culture.

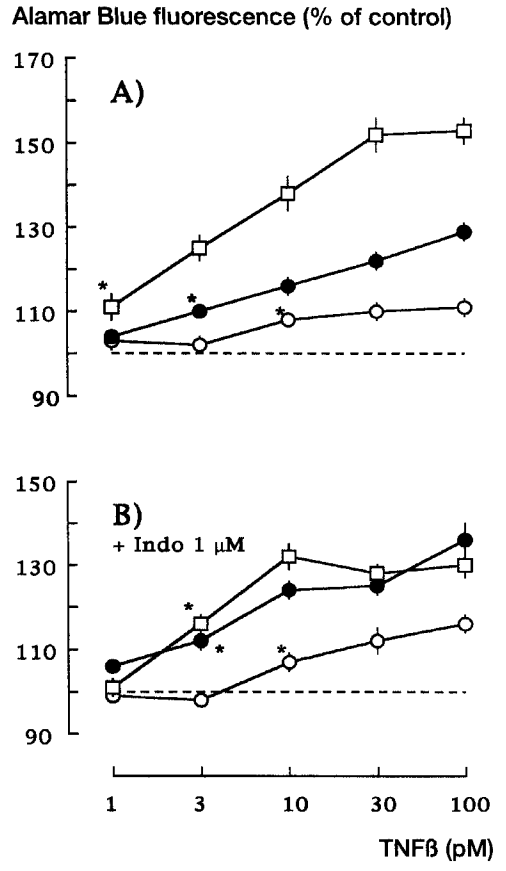

Figure 3. Effect of TNF-ß with (A) or (B) without indomethacin on osteoblastic proliferation. For method, see Figure 1. ○ 4 days in culture, ● 8 days in culture, □ 12 days in culture.

bone eventually formed. A number of cell lines and isolation techniques have been established to study osteoblastic growth. Most of these in vitro systems use animal bone cell cultures, most often rat or murine cell lines from fetal, newborn or young animals. It is not clear whether one can extrapolate results from these systems to adult human bone cells. Transformed human cell lines have been used as a tool for osteoblast studies, although of uniform phenotype, they have unrepressed replication activity and fail to display the normal coupling of differentiation and growth arrest (Quarles et al. 1992). In this study, we used primary cultures of normal human osteoblasts. This is currently believed to be the most reliable cell system reflecting processes occurring in human bone and it is becoming widely used (Marie 1994).

The proliferation rate of cells is most often measured by thymidine incorporation. However, this only reflects cell cycling, while actual cell growth is the sum of cell division and cell death. We use a new technique to measure cell number based on the fluorescent probe Alamar Blue. This dye enters the cells and is reduced in the cellular respiratory chain, thus altering its fluorescent capacity. The changes in fluorescence are directly proportional to the number of cells. This technique has recently been adapted to the study of adherent mesenchymal cells by us, and is described elsewhere (Jonsson et al. 1996). Briefly, it is an atoxic, non-radioactive, one-step procedure, that permits large-scale experiments with long-term cultures.

By combining the isolated human osteoblasts with this technique of measuring cell proliferation, we could detect distinct effects of the inflammatory cytokines IL-1ß, TNF-α and TNF-ß, on osteoblastic proliferation. Our data show marked direct effects of these cytokines on cell proliferation. Although PGE_2 is a potent mitogen for osteoblasts, and all these cytokines were able to enhance PGE_2 formation, the effects on cell proliferation were not dependent on pros-

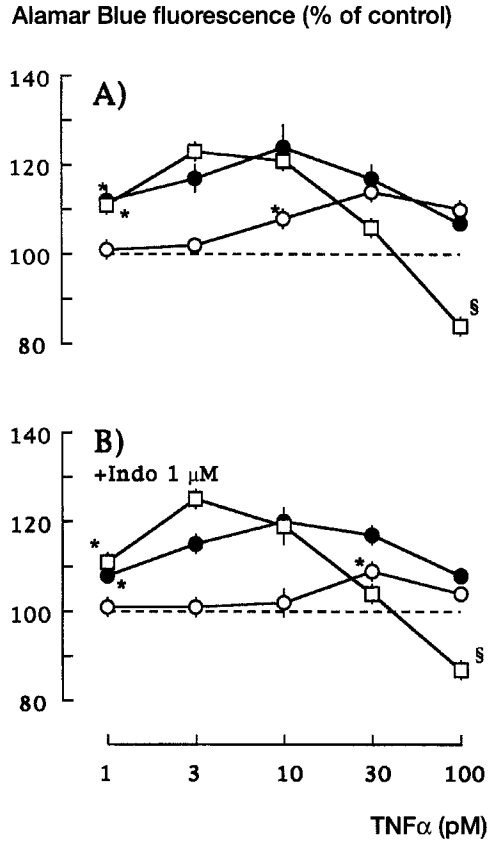

Figure 4. Effect of TNF-α with (A) or without (B) indomethacin on osteoblastic proliferation. For method, see Figure 1. ○ 4 days in culture, ● 8 days in culture, □ 12 days in culture.

taglandin formation. On the contrary, when endogenous prostaglandin formation was blocked by indomethacin, the cells became more sensitive to IL-1ß.

The experiments also revealed that the response to IL-1ß is delayed at least 4 days as compared to TNFs. Thus, IL-1ß and TNF must have different intracellular mechanisms causing cell growth. IL-6, on the other hand, did not affect cell proliferation. The lack of effect by IL-6 is in agreement with data published by Littlewood et al. (1991), who studied the effect in human cells, but in contrast to findings in the rat osteosarcoma cell line UMR, where IL-6 is a mitogen (Fang and Hahn 1991). We could also detect a biphasic response to TNF-α, with a clear inhibition of cell proliferation a higher doses and longer culture time. This effect has not, to our knowledge, been reported before and the mechanism causing it is not known.

In conclusion, our data show that the inflammatory cytokines IL-1ß, TNF-α and TNF-ß enhance the proliferation rate in human osteoblasts, while IL-6 has no effect. Furthermore, higher doses of TNF-α inhibit cell proliferation. Thus, these cytokines have the capacity to regulate osteoblastic proliferation and may therefore be a significant part of the bone-forming processes seen in, e.g., callus formation, in ectopic bone formation and in areas of tumor-induced osteosclerosis.

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