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Increased mRNA Expression and Protein Secretion of Interleukin-6 in Primary Human Osteoblasts Differentiated In Vitro From Rheumatoid and Osteoarthritic Bone

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Abstract We have investigated the expression and synthesis of potential bone-resorbing cytokines, interleukin-6 (IL-6), interleukin-1 (IL-1), and tumor necrosis factor (TNF) in rheumatoid arthritic (RA) and osteoarthritic (OA) bone, two common diseases which are associated with bone loss. Primary human osteoblast (hOB) cultures were established to determine the temporal mRNA expression of IL-6, IL-1 (α and β), and TNF (α and β) in relation to osteoblast growth and phenotypic genes. IL-6 mRNA levels were found to be significantly higher ($P < 0.04$) in both OA hOB (17 patients) and RA hOB (10 patients) compared to normal (NO) hOB (9 patients) and reached five-fold increases in OA hOB and 13-fold increases in RA hOB. Maximal levels of IL-6 are expressed at Day 21 which corresponds to the mineralization stage reflected by decreasing collagen I (α_1), osteopontin, bone sialoprotein, alkaline phosphatase mRNA levels, while osteocalcin (OC) mRNA levels increased. IL-6 protein levels also were significantly higher ($P < 0.05$) in OA hOB and RA hOB compared to NO hOB. These increases were not attributable to sex or age of the donor bone. Neither the mRNA encoding IL-1(α and β) and TNF(α and β) nor the related proteins were detectable. These results indicate that differentiated OA hOB and RA hOB within a bone tissue-like matrix constitutively express and secrete high levels of IL-6. This inherent property suggests that these osteoblasts, independent of local inflammatory parameters, can contribute to enhanced recruitment of osteoclast progenitors and thereby bone resorption. J. Cell. Biochem. 81:666–678, 2001.

Key words: ex vivo; osteoblasts; rheumatoid arthritis; osteoarthritis; interleukin-6; interleukin-1; tumor necrosis factor α

Rheumatoid arthritis (RA) is a chronic inflammatory disease with articular and systemic manifestations. To date symptoms, including periarticular bone loss and generalized osteoporosis, have been related to immunologic processes within the synovial tissue resulting in release of high levels of interleukin-1 (IL-1), tumor necrosis factor (TNF), and interleukin-6 (IL-6) into the synovial fluid with subsequent diffusion to the periarticular bone space [Houssiau et al., 1988; Kotake et al., 1996]. It is generally accepted that periarticular increased bone resorption is the result of the stimulation of osteoclasts by these cytokines. However, it remains unclear whether the generalized bone loss is a consequence of treatment, immobility, or disease activity.

Several studies have reported pathological changes in the bone marrow of patients with RA. Findings, such as elevated levels of IL-6 and interleukin-8 (IL-8) in iliac bone marrow serum [Tanabe et al., 1994] and functionally abnormal CD14+ cells from bone marrow precursors

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[Hirohata et al., 1995], indicate disease related changes in soluble factors and cellular components that may contribute to the pathology of RA. Preliminary results showed that osteoclastogenesis was increased in bone marrow cell cultures from RA patients [Toritsuka et al., 1997] suggesting that increased bone resorption was mediated at the cellular level.

Osteoclast-mediated bone resorption is osteoblast-regulated via several molecular pathways (reviewed by Suda et al., 1999). Osteoprotegerin ligand OPG-L (also known as RANKL and osteoclast differentiation factor (ODF)) which is expressed on osteoblasts, mediates the signal for osteoclastogenesis through binding to its receptor (RANK, receptor activator of NF-B) on pre-osteoclast lineage cells [Lacey et al., 1998; Yasuda et al., 1998]. This biological activity can be neutralized by osteoprotegerin, a soluble decoy receptor [Simonet et al., 1997]. In addition, osteoblasts along with other cell types produce osteoclast-stimulating factors, among which are IL-1, TNF, and IL-6. To date in situ and in vitro studies have shown that normal human osteoblast-like cells constitutively express a low level of IL-6 mRNA, but not IL-1 and TNF [Gowen et al., 1990; Chaudhary et al., 1992; Birch et al., 1993; Lisignoli et al., 1999] and that stimulation (for example by IL-1β) is necessary to produce high levels of these cytokines [Chaudhary et al., 1992]. However, little is known about the expression of these cytokines in osteoblasts from patients affected with RA [Lisignoli et al., 2000] or osteoarthritis (OA) [Kuliwaba et al., 2000].

In this study, we have examined the presence of constitutive mRNA encoding IL-1(α and β), TNF(α and β), and IL-6 during the differentiation of ex vivo cultured human osteoblasts derived from normal human trabecular bone and from bone affected by either RA or OA. We have also determined the supernatant levels of IL-1(α and β), TNF-α, and IL-6 protein in the three groups and showed that IL-6 expression and secretion occurs and is upregulated in mature osteoblasts from RA and OA patients at significantly higher levels than in osteoblasts from normal patients.

MATERIALS AND METHODS

Clinical Specimens

These studies were approved by the local ethical committee (ethical committee for the Municipality of Copenhagen, no. V92355) and informed consent was obtained from the patients. The RA, OA, and NO patients employed in this study were not matched with regard to age, sex, or medical treatment.

Trabecular bone from the femoral head was obtained from ten patients with RA (eight women aged 18–75, mean 43 years and two men aged 71–77, mean 74 years) who underwent joint replacement surgery. The patients satisfied more than five points of the revised American Rheumatism Association criteria for RA [Arnett et al., 1988] and most of them were treated with salicylates and/or non-steroidal anti-inflammatory drugs (NSAID). Four patients were treated with low-dose glucocorticoids.

Osteoarthritic trabecular bone was obtained from 17 patients (eleven women aged 56–88, mean 75 years and six men aged 70–77, mean 72 years) after knee replacement surgery for osteoarthritis. Most of the patients were treated with salicylates and/or NSAID.

Normal trabecular bone was obtained from nine patients (five females aged 7–69, mean 42 years and four males aged 15–79, mean 47 years) who underwent corrective surgery following accidental injury, or amputation of the lower extremity to remove soft-tissue tumors.

Materials

Tissue culture media and reagents, heat-inactivated fetal calf serum (FCS), trypsin-EDTA (0.05–0.02%, respectively) were all purchased from Bie & Berntsen (Copenhagen, Denmark). Six-well plates, slide flasks and 175 cm^2 tissue culture flasks were purchased from Nunc (Roskilde, Denmark). Guanidine thiocyanate was purchased from Fluka (Ronkonkoma, NY). Phenol water saturated and Litex Agarose HSB 200 were purchased from Medinova (Copenhagen, Denmark). All other chemicals unless otherwise stated were obtained from Sigma (UK). Zeta-Probe GT membrane was purchased from Bio-Rad laboratories (Copenhagen, Denmark). α^{32}P-dCTP and a random-primer labeling extension kit were purchased from Amersham Life Science (Little Chalfont, Buckinghamshire, UK). Quick Spin G-25 Sephadex Columns were purchased from Boehringer (Mannheim, Germany). Pelikine Compact kit was purchased from CLB (Amsterdam, The Netherlands).

Human Bone Cell Cultures

Explants of trabecular bone were cultured using the method described by Beresford et al. [1984] with some modifications. Briefly, the connective tissue and cortical bone were carefully removed from the bone using bone cutters. The remaining trabecular bone was minced into small fragments (3–5 mm in diameter), which then were extensively rinsed with sterile saline buffer to remove blood and marrow. The bone fragments were seeded as explants in 175 cm^2 tissue culture flasks, 10–15 fragments per flask and cultured in Earles minimum essential medium (MEM) supplemented with 10% (v/v)-heat-inactivated fetal calf serum (FCS), 50 μg/ml ascorbic acid, 2 mM L-glutamine, and non essential amino acids. Sufficient medium was added to keep the explants moist (50–60 ml). All the medium was changed at 48 h and thereafter once a week with only half the medium being changed. Cultures were established by outgrowth from explants incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for at least three weeks. Cells cultured by this procedure have been characterized previously and shown to possess an osteoblast-like phenotype [Beresford et al., 1986]. This includes the production of cyclic AMP in response to PTH, synthesis of collagen type I, alkaline phosphatase, and osteocalcin. At preconfluence, bone explants were removed and cells were detached, using trypsin-EDTA. Cells from the same patient were pooled and plated at a density of $3.5\,10^6$ cells/flask in 175 cm^2 tissue culture flasks where the cells were allowed to grow. Only first-passage cells were used in the experiments and cultured in the medium described above. Every third day half of the medium was renewed and the conditioned medium was then collected and frozen at −70°C until analysis. Antibiotics (penicillin–streptomycin, 100 U/ml and 100 μg/ml, respectively) were only added to the cultures twice, initially and at the time of trypsination.

At the time the cell strains were subcultured, samples of the cell suspensions were plated in a slide flask, allowed to settle overnight, and examined for contaminating cells of the lymphoid and monocytic lineage (a potential source of cytokines) by specific immuno-staining with CD14 mABS (Becton & Dickinson, Basel, Switzerland). No positive staining was detected (data not shown) which is in accordance with others studies using a panel of monoclonal antibodies [Skjodt et al., 1989], examining morphology (Wright-Giensa staining) and non-specific esterase staining [Li et al., 1973].

Morphology and Histochemistry

Parallel subcultures in six-well plates were monitored by observation in the light microscope after having been stained with toluidine blue, alkaline phosphatase, and von Kossa following standard procedures [Humason, 1967]. Growth pattern and changes during cultivation were recorded.

Total Cellular RNA Preparation and Analysis

For each time point, cells were pooled from 3×175 cm^2 flasks and total cellular RNA was purified by a single-step acidified guanidinium thiocyanate phenol extraction method [Chomczynski and Sacchi, 1987]. RNA preparations were quantitated and purity assessed by ultraviolet spectroscopy at 260 and 280 nm and equal amounts of RNA (10 μg in the case of IL-6 studies and 15 μg in the case of IL-1,TNF, and phenotypic markers studies) were size fractionated by electrophoresis on 1% agarose–6.6% formaldehyde gels [Lehrach et al., 1977]. RNA intactness was assessed by ethidium bromide staining and RNA was transferred to Zeta-Probe membranes by 3 h downward alkaline transfer. Prehybridizations and hybridizations were performed in a solution containing 50% formamide and at 42°C for 1h and overnight, respectively. DNA probes were labeled with α^{32}P-dCTP by the random primer method. Filters were washed to a final stringency of 0.1% SSC at room temperature, except in the case of hybridizations with human 18S ribosomal DNA probe where the washing temperature was 65°C. Filters were exposed 3 days (overnight for human 18S ribosomal DNA probe) and signals were quantitated by a PhosphorImager (Fuji Medical Systems, Stamford, CT). The data presented in arbitrary photo-stimulation luminescence (PLS) units were normalized to signals obtained after hybridization with the 18S ribosomal gene probe to correct for variations in gel loading and transfer.

cDNA Probes

Purified inserts were used as DNA probes. Plasmids containing a cDNA probe for the human IL-1α gene (insert 0.65 kbp, HindIII/HindII) and the human IL-1β gene (insert 0.65

kbp, EcoRI/PstI) were generous gifts from Dr. Steven Gillis (Immunex Corporation, Seattle, WA). The plasmid containing a cDNA probe for the human IL-6 gene (insert 0.44 kbp, TaqI/BanII) was a generous gift from Dr. T. Hirano (Osaka University, Osaka, Japan). A plasmid containing a cDNA probe for the human TNFα gene (insert 0.82 kbp, EcoRI) was a generous gift from Arjun Singh (Genentech, South San Francisco, CA). The plasmid containing a cDNA probe for human TNFβ (insert 0.53 kbp, BamHI/HindIII) was purchased from British Biotechnology, Abingdon, England. Human histone H4 cDNA probe was obtained from plasmid pUC8 (insert 2.3 kbp, PstI/BamHI) [Collart et al., 1991]. cDNA probe for human proα1(I) collagen was derived from plasmid pUC8 (insert 0.67 kbp, EcoRI/HindIII) which was kindly provided by Dr. Eero Vuorio (University of Turku, Finland). Human BSP cDNA was a generous gift from Dr. Larry W. Fisher (National Institute of Dental Research, MD). Human OP cDNA probe was derived from plasmid pOP10 (1.5 kbp, XhoI-XbaI) [Young et al., 1990]. Probes were obtained for human AP cDNA [Noda et al., 1987], human OC cDNA [Celeste et al., 1986], and human 18S rDNA [Wilson et al., 1978].

Complementary DNA probes were labeled to approximately 10^6 dpm/μg with α^{32}P-dCTP using a random priming protocol [Feinberg and Vogelstein, 1983]. Unincorporated label was separated from labeled probe with a Quick Spin™ column (Boehringer Mannheim Corporation, Indianapolis, IN) as described by the manufacturer.

ELISA for IL-6

For each cell culture investigated, equal samples of conditioned media from three culture flasks were collected at the times indicated (7, 10, 16, 21, 26, and 30 days) during the differentiation time course of hOB. The samples were pooled and frozen at −80°C until analysis by an enzyme-linked immunosorbent assay (ELISA) specific for human IL-1 (α and β), TNF-α, and IL-6. The assay was performed as previously described [Hansen et al., 1991] using 100 μl of standard or sample volume per well in 96-well microplates. The inter-and intra-assay coefficients of variation for the concentration range between 10 pg/ml and 0.01 pg/ml were less than 15%. The sensitivity-limits of these ELISAs were 8–30 pg/ml. The protein levels were determined in duplicate and corrected for the protein in FCS, since culture medium was supplied with 10% FCS.

Statistics

All statistical differences were determined using the unpaired Student's *t*-test. In the figures, one asterisk (*) denotes a significant difference, $P < 0.05$.

RESULTS

Developmental Stages of RA, OA, and NO Human Osteoblasts During Culture

At selected time points throughout a 30 day culture period, morphologic and histochemical examination of the human osteoblasts (hOB) isolated from each group showed a progressive development towards a bone tissue-like organization, exhibiting nodule formation and extracellular matrix mineralization beginning on Day 21. No significant differences were observed among the three groups. By Day 14, approximately 80% of the cell layer was alkaline phosphatase positive. Figure 1 shows a representative example of cell morphology from an RA hOB culture after 14 and 21 days (Fig. 1B and D, toluidine blue) with alkaline phosphatase positive cells throughout the well and in multilayered nodule areas (Fig. 1A and C). The distribution of mineral deposits throughout the extracellular matrix is shown on Day 30 (Fig. 1E and F). The extent of mineralization reflects the absence of β-glycerol 2-phosphate in the media used in these studies.

These morphological and histochemical evidences showing osteogenic differentiation are further supported by Northern blot analysis for the expression of genes that are transcribed during the growth and maturation stages of osteoblast phenotype development. Expression of the cell growth-associated H4 histone gene revealed that proliferation was maximal during the first 7–10 days in culture regardless of the disease affecting the bone donor. Thereafter, cell growth decreased slowly through Day 21 (Fig. 2A). The mRNAs of osteoblast phenotypic genes we examined followed the same temporal pattern of expression in the three groups (Fig. 2B). The expression of collagen type I, osteopontin, bone sialoprotein, and alkaline phosphatase decreased. Osteocalcin mRNA levels increased from Day 14 to 21 as the cultures progressed into the mineralization stage.

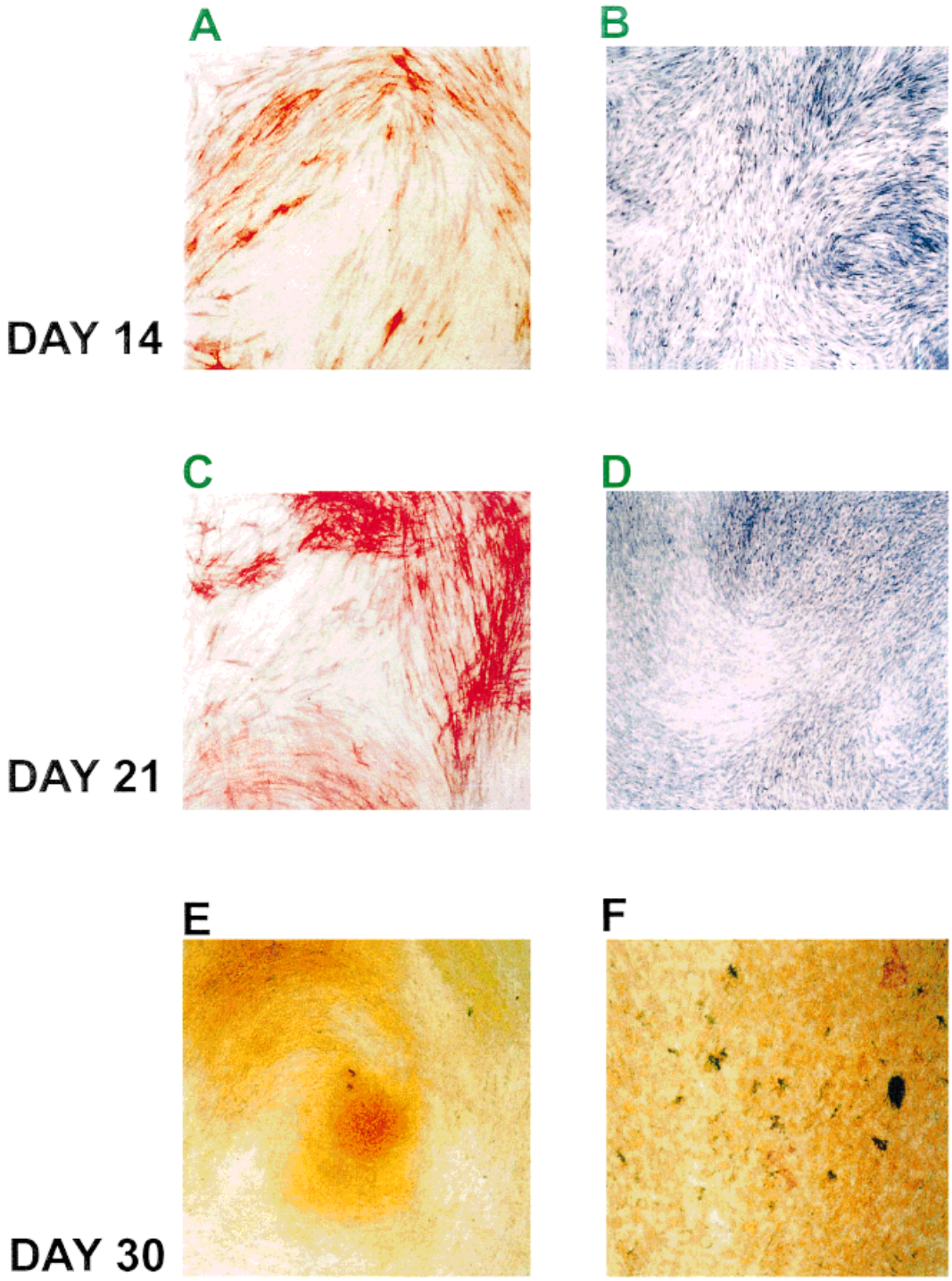

Fig. 1. Morphologic and histochemical examination of primary RA hOB culture: nodule formation, alkaline phosphate activity, and mineralization. All osteoblast cultures were maintained in MEM media with 10% FCS, 50 μg/ml ascorbic acid. Procedures were performed, as described in Materials and Methods. **A**, **C:** Alkaline phosphatase staining showing positive stained cells throughout the culture dish (Day 14 and 21) prominent in multilayered areas. **B**, **D:** Toluidine blue staining showing the cellular multilayering that initially develops the areas or nodules in which mineralization initiates (Day 14 and 21). **E:** von Kossa staining demonstrating mineral deposition (Day 30) in multilayered regions (A–E at 40X) and **F:** at higher magnification 80X showing focal deposits of mineral with accumulation of the extracellular matrix (Day 30).

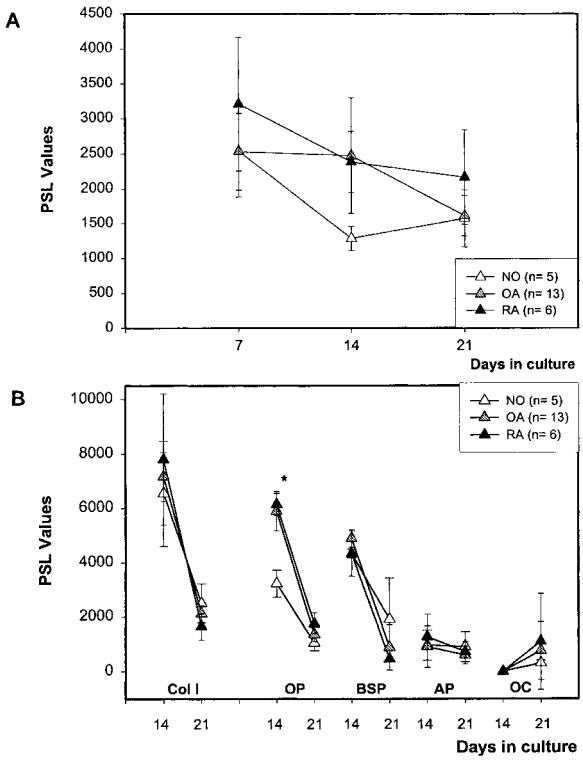

Fig. 2. Expression of cell growth-, extracellular matrix-, and osteoblast phenotype-related genes during the development of hOB in vitro. Samples of total RNA isolated at the times indicated (7, 14, and 21 days) during the differentiation time course, were assayed for the steady state levels of various transcripts by Northern blot analysis. The resulting signals were quantitated using a Phosphorimager and mRNA levels are expressed relative to 18S rRNA levels. **(A)** shows mean ± SD of histone H4 mRNA during the proliferation and differentiation of primary hOB cultures derived from NO bone (n = 5), OA bone (n = 13) and RA bone (n = 6). **(B)** shows mean ± SD of collagen I (α_1) (COL I), osteopontin (OP), bone sialoprotein (BSP), alkaline phosphatase (AP) and osteocalcin (OC) during differentiation of primary hOB on Days 14 and 21.

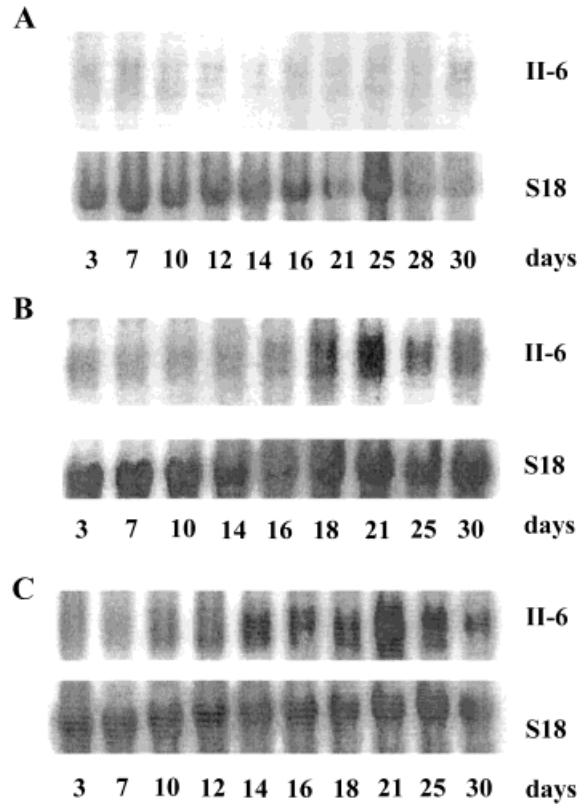

Fig. 3. Expression (mRNA) of IL-6 by osteoblasts from a representative normal **(panel A)**, osteoarthritic **(panel B)** and rheumatoid arthritic **(panel C)** patient. The cells were cultured for 30 days. Total cellular RNA was prepared at the indicated days and 10 μg were loaded per lane for each gel. The Northern blot was hybridized sequentially with IL-6 and 18S rRNA probes. This experiment was repeated at least two times for each cell culture.

We note that osteopontin mRNA levels appear two-fold higher on Day 14 in the OA and RA groups compared to the NO controls (Fig. 2B). Histone H4 mRNA levels also appear higher in the patient groups compared to NO (Fig. 2A), although the differences are not statistically significant. The over all temporal expression and magnitude of histone H4 mRNA is similar indicating that cellular proliferation occurs at the same rate in the three groups.

Expression Levels of IL-6, IL-1(α and β) and TNF(α and β) During the Developmental Stages of hOB In Vitro

The mRNA levels of the five cytokines IL-6, IL-1(α and β), and TNF(α and β), were examined at selected time points throughout the course of osteoblast growth and differentiation during thirty days of cultures. Representative Northern blots show that the IL-6 mRNA transcript was detected as a single 1.3 kb band in all three groups of hOB (28/28 patients) (Fig. 3). The mRNA transcripts for IL-1(α and β) and TNF(α and β) were not detected except for one of the OA cultures which was accidentally contaminated by bacteria in these groups. In this culture, we could detect an IL-1β and IL-1α transcript as a single 1.9 kb and 2.1 kb band, respectively (data not shown). This particular OA cell culture was excluded from further analysis.

Figure 4 compares the temporal expression of normalized IL-6 mRNA during osteoblast differentiation from the Northern blot hybridizations for the three groups. During the developmental stages of hOB, IL-6 is expressed in the normal hOB at maximal levels during the

growth period and thereafter at constitutive low levels. When OA and RA hOB were compared to NO hOB (Fig. 4A), the levels of IL-6 mRNA were significantly ($P < 0.04$) higher in OA and RA hOB in the post-proliferative differentiation stages. Moreover, the increased level of IL-6 mRNA in the RA group was 3–4 fold greater when compared to OA hOB. The significant increases occurred at a time which corresponds to the peak of IL-6 expression levels in the differentiated osteoblast phenotype (reflected by OC mRNA, see Fig. 2B). During the 30 day culture period in the RA and OA patient groups, IL-6 mRNA expression continually increased reaching a peak at Day 21. In the RA group, IL-6 mRNA remained 9-fold elevated compared to NO hOB throughout the culture period. Compared to the NO group, maximal levels of IL-6 expression reached a 13-fold increase in RA and a 5-fold increase in OA. Notably, mRNA expression for the other cytokines was undetectable (data not shown).

Because IL-6 has been related to bone loss induced by estrogen deficiency [Girasole et al., 1992], we have compared the levels of IL-6 mRNA as a function of bone donor sex (Fig. 4B) and age (Fig. 4C). No significant differences attributable to the sex or age of the bone donor were found. Therefore, the observed IL-6 mRNA expression in the patient groups does not increase with age and is not more elevated in females compared to males. Moreover, the temporal expression pattern was the same, independent of age and sex, as IL-6 mRNA levels gradually increased to a maximum around Day 21 which corresponds to fully differentiated osteoblasts in a mineralized matrix.

IL-6, IL-1(α and β) and TNF-α Protein Production by hOB

To determine whether increased IL-6 mRNA levels were accompanied by increased IL-6 protein, constitutive production of immunoreactive IL-6 by hOB was assessed by measuring IL-6 concentrations in the conditioned medium of representative samples from each group using an IL-6 specific antibody in an ELISA (Fig. 5). The concentration of IL-6 in medium containing 10% FCS was above the detection limit of the assay of < 30 pg/ml. IL-6 production correlated with expression of IL-6 mRNA. At Day 16, IL-6 production was significantly ($P < 0.04$) higher in OA and RA hOB

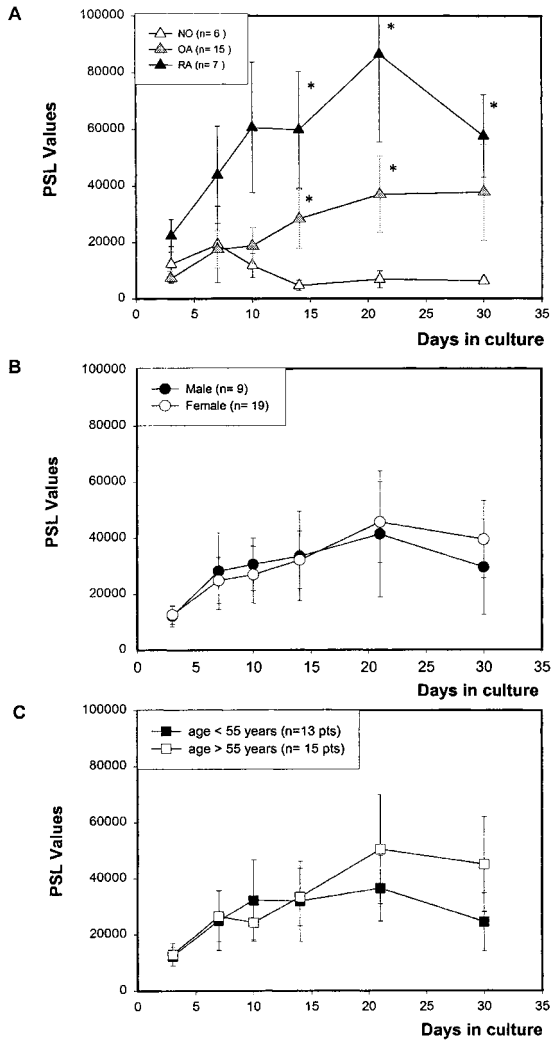

Fig. 4. Time course of increased IL-6 mRNA levels in RA and OA human osteoblasts compared to normal during osteoblast development in vitro. Cells derived from trabecular bone of RA, OA, and NO patients were cultured after confluence and total cellular RNA was isolated at the times indicated (3, 7, 10, 14, 21, and 30 days) during the differentiation time course. IL-6 mRNA levels were determined by northern blot hybridization using human IL-6 and 18S rRNA cDNA probes. Quantification of the signals were made by PhosphorImager in photostimulation- luminescence (PSL). 18S rRNA was used for control for the equivalence of loading, therefore IL-6 mRNA levels are expressed relative to 18S rRNA levels. **(A)** shows mean ± SD of PhosphorImager measurements of the constitutive IL-6 mRNA during the differentiation of separate primary human osteoblast cultures derived from normal bone (n = 6), osteoarthritic bone (n = 15) and rheumatoid arthritic bone (n = 7). **(B)** shows the same set of measurements when related to the sex of the patients (female (n = 19) and male (n = 9)). **(C)** shows the same set of measurements when related to the age of the patients and the onset of menopause (age < 55 years old (n = 13) and age ≥55 years old (n = 15)).

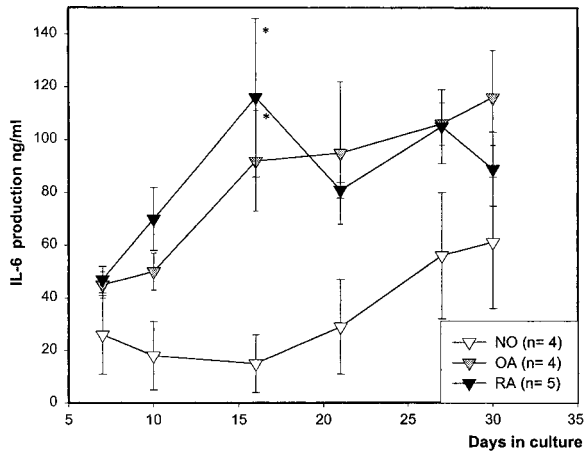

Fig. 5. Increased constitutive IL-6 production by RA and OA hOB cells in culture compared to normal hOB. hOB cell-conditioned media were collected in relation to the every third day cell feeding. For each cell culture, samples from three flasks were pooled at the times indicated (7, 10, 16, 21, 27, and 30 days) during the differentiation time course and assayed for IL-6 by ELISA. Values represent mean (± SD) of RA (n = 5), OA (n = 4) and NO (n = 4) hOB cell cultures.

reaching a maximal eight-fold increase in RA hOB and a six-fold increase in OA hOB compared to NO hOB.

Because the lack of detectable mRNA for IL-1(α and β) and TNF(α and β) could be due to unstable mRNA, we also measured the concentration of IL-1(α and β) and TNF-α proteins. All three proteins were undetectable in the same media in which we observe accumulation of IL-6 (Fig. 6).

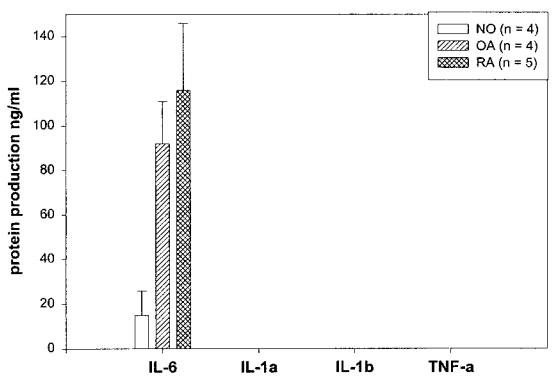

Fig. 6. Constitutive IL-6, IL-1α, IL-1β, and TNF-α production by RA, OA, and normal hOB at Day 16 of cell culture. For each cell culture, samples from three flasks were pooled and assayed for IL-6, IL-1α, IL-1β, and TNF-α by ELISA. Values represent mean (± SD) of RA (n = 5), OA (n = 4) and NO (n = 4) hOB cell cultures.

DISCUSSION

In this study we examined the spontaneous production of bone resorbing cytokines at both RNA and protein levels in primary osteoblast cultures derived from trabecular bone of RA, OA, and NO patients. We found that osteoblasts from all three groups express IL-6 mRNA and secrete IL-6 protein as opposed to IL-1(α and β) and TNF(α and β) whose mRNAs and related proteins were undetectable. Our results show for the first time enhanced constitutive level of IL-6 mRNA during the differentiation of both RA and OA hOB in vitro compared to NO. The constitutive IL-6 mRNA expression was markedly increased in RA hOB and only increased to a lesser extent in OA hOB by Day 21. Others have demonstrated a correlation between cytokine mRNA expression and protein synthesis [Littlewood et al., 1991]. This correlation was confirmed by our results since we found that the increasing IL-6 mRNA expression over time in diseased hOB was paralleled by the secretion of the protein IL-6. The progressive development of a bone tissue-like organization, consisting of multilayered nodules in an ordered, mineralized collagen extracellular matrix, is preserved reflecting the similar expression of bone markers among the RA, OA, and NO hOB groups despite the high level of IL-6 protein secreted into the culture media in the patient groups. The five-fold and 13-fold higher levels of IL-6 mRNA in OA and RA groups did not stimulate DNA synthesis or decrease associated gene expression of osteoblast matrix forming proteins. Indeed, elevated osteopontin mRNA in the OA and RA groups is consistent with a previously observed increase in osteopontin by IL-6 in a leukemia cell line [Franchimont and Canalis, 1995; Matsumoto et al., 1998].

The absence of basal levels IL-1 and TNF-α expression at any stage of osteoblast differentiation is consistent with previous reports. It is well established that osteoblasts can be stimulated by local bone-resorbing agents such as IL-1(α and β), TNF-α, and LPS (reviewed in Roodman, 1992; Manolagas, 1995); [Feyen et al., 1989; Ishimi et al., 1990; al Humidan et al., 1991; Linkhart et al., 1991; Littlewood et al., 1991] and by systemic factors such as parathyroid hormone (PTH) [Feyen et al., 1989; Lowik et al., 1989] to produce high levels of IL-6, IL-1(α and β), and TNF-α [Gowen et al., 1990]. Only in one case, i.e. an OA cell culture accidentally

contaminated by bacteria, mRNA for IL-1(α and β) became measurable. This is in accordance with previous in vitro studies where IL-1β and TNF-α transcripts could only be identified in stimulated hOB [Gowen et al., 1990; Keeting et al., 1991; Linkhart et al., 1991; Merry and Gowen, 1992] or by PCR [Birch et al., 1993].

In contrast to these stimulated responses in cytokine expression, inconsistent observations have been made concerning the production of IL-6 mRNA and protein in unstimulated osteoblasts. Studies performed in serum-free conditions (1mg/ml bovine serum albumin (BSA)) reported no detectable mRNA expression and no or very low IL-6 production [Linkhart et al., 1991; Bertolini et al., 1994], whereas our study and others [Littlewood et al., 1991; Franchimont and Canalis, 1995] performed with FCS in the conditioned medium showed a spontaneous IL-6 production. Littlewood and coworkers showed that by increasing the serum content in the medium, IL-6 production was enhanced [Littlewood et al., 1991]. Among the constituents of FCS, growth factors have been shown to stimulate IL-6 production [Elias et al., 1991; Manolagas et al., 1994, Franchimont and Canalis, 1995]. This could explain why we and others were able to measure a constitutive IL-6 production in hOB when cultured in the presence of serum. The length of the incubation time preceding the removal of cell-conditioned medium seem also to influence the results as studies based on short time incubation (for example 12h and 24h) could not detect any basal mRNA expression by Northern analysis [Linkhart et al., 1991; Kassem et al., 1996] nor IL-6 production [Linkhart et al., 1991]. The basal production of IL-6 by normal osteoblasts being low compared to the production following stimulation, longer incubation times seems to be necessary considering the detection limit of the assay used.

Previous studies on synovial fibroblasts [Guerne et al., 1989, Bucala et al., 1991; Seitz et al., 1994] and chondroblasts [Bender et al., 1990, Guerne et al., 1990; Nietfeld et al., 1990] from humans have shown a substantial level of constitutive IL-6 production. In one of the studies [Bucala et al., 1991], i.e. on synovial fibroblasts, the investigators reported increased production of IL-6 in rheumatoid synovial fibroblasts, but this was based on only one cell line. All the other studies cited here failed to find significant differences in the amounts released between osteoarthritic, rheumatoid arthritic and normal cells. So at present and at least in in vitro experiments, it appears that high constitutive IL-6 production is unique to osteoarthritic and rheumatoid arthritic human osteoblasts.

A central question posed by our findings regards the mechanism by which RA and OA osteoblasts can be induced to undergo such prolonged phenotypic change that result in enhanced constitutive IL-6 production. It has been postulated that one possibility for stimulated IL-6 osteoblast secretion is that the infiltration of activated inflammatory cells and local production of monocytic and lymphocytic inflammatory products creates an appropriate milieu that drives osteoblasts into a long-term, activated state. However, the nature of such inducing products as well as the mechanism by which activation occur remain to be clarified [Bucala et al., 1991; Rifas and Avioli, 1999]. Constitutive differences in the responsiveness of osteoblasts to inflammatory mediators may exist, thus rendering some individuals more susceptible to the sequelae of chronic arthritis.

Studies in both human and murine models suggest that IL-6 is regulated by sex steroids [Girasole et al., 1992; Passeri et al., 1993; Poli et al., 1994; Bellido et al., 1995; Lin et al., 1997]. However, while some studies conducted on human osteoblast cell line models have not confirmed this [Chaudhary et al., 1992; Rifas et al., 1995; Pacifici, 1996],other findings suggest that age and estrogen status influence either IL-6 basal or stimulated levels in bone cells. Stimulated production of IL-6 was inhibited by 17β-estradiol in human fetal osteoblast-like cell line in which the ER gene was expressed at high levels [Kassem et al., 1996]. Stromal cells [Cheleuitte et al., 1998] from women receiving estrogen replacement therapy showed significantly lower basal secretion of IL-6. Thus estrogens can modulate IL-6 production in hOB, dependent on the stage of osteoblast differentiation and levels of ER [Bodine et al., 1997]. However, divergent results have been observed and may be due to differences between rodent and hOB, as well as the low and variable numbers of estrogen receptors (ER) in the hOB cell lines used.

In our study, a small amount of estrogen/androgen is likely present in the FCS [Rickard et al., 1992] added to culture medium, which may have mediated biological effects. No

attempt was made to evaluate the number of estrogen receptors in the hOB cells. Although the RA group was almost entirely female, no significant differences in IL-6 mRNA levels were found when patients age and sex were compared. Moreover the temporal expression pattern of IL-6 mRNA was the same showing a steady enhancement throughout the developmental stages of differentiating osteoblasts and peaking around the matrix-secretion stage at Day 21. This pattern is in accordance with in situ hybridization studies on developing human bone [Dodds et al., 1994] that showed IL-6 mRNA expression within pre-osteoblasts and in newly differentiated and matrix-secreting osteoblasts, but absent or reduced in flattened, inactive osteoblasts. Together, these findings support our conclusion that high constitutive levels of IL-6 in hOB are truly related to RA and OA and not related to the age or the sex of donors.

The question arises as to the in vivo significance of this particular enhanced IL-6 expression and production in OA and RA hOB. Recently, in vivo histomorphometric studies in athymic mice indicated an increase in both bone formation parameters and osteoclast activity, suggesting IL-6 modulates bone turnover [Rozen et al., 2000]. IL-6 has been suggested to mediate osteoblast pro-differentiating [Bellido et al., 1997] and anti-apoptotic effects in human osteoblastic cells [Bellido et al., 1998]. Thus higher constitutive levels in differentiated osteoblasts in mineralizing matrix from OA and RA bone, not stimulated by exogenous cytokines, may be the result of a compensatory mechanism to preserve osteoblasts in the milieu of high bone turnover. However, IL-6 has been shown to be a potent stimulator of the development of osteoclasts from their hematopoietic progenitors by acting on osteoblasts to induce OPG-L, the osteoclast differentiation factor (reviewed in Suda et al., 1999). Increased production of IL-6 has been implicated in the pathophysiology of many different disease states characterized by increased bone resorption, including postmenopausal osteoporosis and RA [Manolagas et al., 1996]. Consequently, a strong and prolonged constitutive IL-6 production by OA and especially by RA osteoblasts as found in this study would support the critical role of IL-6 in sustaining a continuous influx of osteoclasts from their progenitors. Thus, IL-6 elevated basal expression in osteoblasts from OA and RA diseases may have dual roles in contributing to bone turnover.

CONCLUSION

In summary, our data are consistent with the notion that IL-6 is a signal for osteoclast formation as compared to IL-1 and TNF and that IL-6 stimulation of bone resorption may be, at least in part, a result of increased IL-6 production by hOB. Besides the pathogenetic role played by IL-6 in the bone loss caused by gonadal deficiency, our results suggest that IL-6 is also a pathogenetic factor in the bone resorption associated with RA. The enhancement of IL-6 expression seen in osteoarthritic hOB was less pronounced and the significance of this finding remains to be elucidated. Our findings emphasize the role of IL-6 in RA and OA thus rendering IL-6 signal transduction pathways targets for therapy of chronic inflammation.

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