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J Immunol (2002) 168 (10): 5310–5317.
https://doi.org/10.4049/jimmunol.168.10.5310

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Inflammatory Microcrystals Alter the Functional Phenotype of Human Osteoblast-Like Cells In Vitro: Synergism with IL-1 to Overexpress Cyclooxygenase-2[1]

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Chronic crystal-associated arthropathies such as gout and pseudogout can lead to local bone destruction. Because osteoblasts, which orchestrate bone remodeling via soluble factors and cell-to-cell interactions, have been described in contact with microcrystals, particularly in uratic foci of gout, we hypothesized that microcrystals of monosodium urate monohydrate (MSUM) and of calcium pyrophosphate dihydrate (CPPD) could alter osteoblastic functions. MSUM and CPPD adhered to human osteoblastic cells (hOB) in vitro and were partly phagocytized as shown by scanning electron microscopy. MSUM and CPPD dose-dependently stimulated the production of PGE_2 in hOB as assessed by enzyme immunoassay, a response that was synergistically enhanced in the presence of IL-1. The mechanism of this synergism was, at least in part, at the level of the expression of cyclooxygenase-2 as evaluated by immunoblot analysis. MSUM and CPPD also stimulated the expression of IL-6 and IL-8 and reduced the 1,25-dihydroxyvitamin D_3-induced activity of alkaline phosphatase and osteocalcin in hOB (with no synergism with IL-1). MSUM- or CPPD-stimulated expression of IL-6 in hOB pretreated with the selective cyclooxygenase-2 inhibitor NS-398 was increased, unlike that induced by IL-1 alone which was partially reduced. MSUM-, CPPD- or IL-1-induced expression of IL-8 was unchanged by pretreating hOB with NS-398. These results suggest that inflammatory microcrystals alter the normal phenotype of hOB, redirecting them toward reduced bone formation and amplified osteoblast-mediated bone resorption, abnormalities that could play a role in the bone destruction associated with chronic crystal-induced arthritis. ***The Journal of Immunology*****, 2002, 168: 5310–5317.**

The long term evolution of chronic inflammation of joints is, in most cases, associated with local destruction of cartilage, ligaments and bone, leading to major and often irreversible locomotor handicaps. Chronic gout is an example of such an inflammatory process which is accompanied by histopathologically characteristic major bone destruction. Two types of tissue lesions are associated with monosodium urate monohydrate (MSUM)[3] microcrystals: 1) some MSUM foci from adjacent joints and cartilage are surrounded by an inflammatory tissue with giant multinucleated cells and macrophages; 2) other uratic foci are described without inflammatory tissue and are "located in the immediate subchondral bone and even those present deeper in the spongiosa undoubtedly form there as independent deposits resulting from the direct deposition of urates in the bone marrow" (1). This histological pattern suggests direct interactions between MSUM microcrystals and bone cells. In contrast, chronic pseudogout, the causal agent of which are calcium pyrophosphate dihydrate (CPPD) microcrystals, is also, albeit very rarely, associated with destructive bone lesions different from those observed in gout (2). In vitro, microcrystals strongly activate inflammatory cells such as monocytes-macrophages and neutrophils to express IL-1, a powerful osteotropic cytokine inducing bone resorption (3, 4). Additionally, IL-1 is up-regulated whereas IL-1Ra, the natural antagonist of the IL-1 receptor, is down-regulated by inflammatory microcrystals in human neutrophils, leading to a significant increase of the bioactivity of IL-1 (5). The osteoclast-activating functions of IL-1 and the associated bone resorption require intermediate osteoblastic factors (6, 7). One of the osteoblastic factors implicated in bone resorption is PGE_2 (8, 9). In fact, excessive production of PGE_2 has been associated with bone loss in human diseases such as rheumatoid arthritis (10), and the down-regulation of cyclooxygenase-2 (COX-2)-dependent PGE_2 synthesis has been related to the suppressive effects of IL-13 or IL-4 on bone resorption (11). Catabolic functions of PGE_2 in bone are directly mediated through actions on osteoclasts, and indirectly through increases of osteoclast recruitment (12, 13). Moreover, the major source of prostaglandins in bone is osteoblastic cells, and PGE_2 represents the predominant arachidonic acid-derived metabolite in various experimental conditions of stimulation of human osteoblast-like cells (14).

Osteoblasts are essential cellular components of the bone remodeling unit active through the expression of soluble factors and cell-to-cell interactions. They are also capable of phagocytosis of different solid particles as demonstrated by microscopy (15, 16). Phagocytizing osteoblasts are activated in vitro, leading to a reduction of cell proliferation and of the production of type I collagen and alkaline phosphatase, and to the increased formation of

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Received for publication October 12, 2001. Accepted for publication March 13, 2002.

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[1] This work was supported by grants from the Canadian Institutes for Health Research, the Canadian Arthritis Network, and The Arthritis Society.

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[3] Abbreviations used in this paper: MSUM, monosodium urate monohydrate; CPPD, calcium pyrophosphate dihydrate; COX-2, cyclooxygenase-2; CPPD, calcium pyrophosphate dihydrate; hOB, human osteoblast-like cell; LDH, lactate dehydrogenase; EIA, enzyme immunoassay; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; AChE, acetylcholinesterase.

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PGE_2 and osteotropic cytokines such as IL-6 (17–19). Human osteoblasts are also strongly stimulated by IL-1 to generate cytokines (14, 20, 21). Thus, IL-1 appears to be the most powerful stimulator of the expression of IL-6 mRNA and bioactivity by human osteoblast-like cells in vitro (22). Moreover, the expression of IL-6 by osteoblasts was shown to be up-regulated by PGE_2 (23), and the proinflammatory cytokine IL-1 represents a strong activator of PGE_2 accumulation in osteoblasts (24). IL-1 also stimulates the expression of IL-8 by human osteoblasts (21), a powerful chemoattractant for neutrophils. Taken together, these various lines of evidence suggest that osteoblasts in direct contact with inflammatory microcrystals could play a role in the local bone resorption associated with chronic crystal-induced arthritis.

In the present study, we tested the hypothesis that inflammatory microcrystals such as MSUM and CPPD microcrystals alter important functions of human osteoblast-like cells such as the expression of PGE_2, IL-6 and -8, alkaline phosphatase, and osteocalcin and that the presence of IL-1 modifies the response of human osteoblast-like cells to these microcrystals.

Materials and Methods

Reagents

αMEM, calcium-free MEM, and penicillin/streptomycin were from Life Technologies (Gaithersburg, MD). FBS was from HyClone Laboratories (Logan, UT). FMLP, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and colchicine were purchased from Sigma-Aldrich (St. Louis, MO). NS-398 was from Biomol Research Laboratories (Plymouth Meeting, PA). Recombinant human IL-1β (IL-1 used in the following text will refer to this specific protein) was a gift from Dr. A. Shaw (Biogen, Geneva, Switzerland) and contained <5 pg/ml endotoxin. Rabbit polyclonal Ab anti-COX-2 was a gift from Dr. J. Grassi (CE-Saclay, Gif-sur-Yvette, France). It was prepared against the peptide (C)-NASSSRSGLDDINPTVLLK present only in the C terminus of human COX-2, and its specificity was previously characterized (25). Triclinic microcrystals of MSUM and CPPD were prepared under sterile pyrogen-free conditions according to a previously described method (26). The mean sizes of the microcrystals used in this study, as determined by scanning electron microscopy, were 10×1.25 and 12×2.8 μm for MSUM and CPPD microcrystals, respectively.

Culture of human osteoblast-like cells (hOB)

hOB were obtained as previously described (27). Briefly, human bone cells were isolated from trabecular bone of surgical pieces obtained during hip or knee replacements. Bone fragments were washed extensively and vigorously in PBS to remove blood cells and were cleaned to remove adhesive soft tissues. These bone fragments were further cut into small pieces, and three to five bone explants were seeded in six-well culture plates. hOB were grown in αMEM supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2. The medium was replaced every 3–4 days until cellular confluence. At confluence, bone explants were transferred to new six-well plates to allow remaining hOB to migrate and adhere to the plate. Human OB were recovered using trypsin-EDTA and plated at starting densities of $0.5–1 \times 10^6$ cells/well in αMEM with 10% FBS. All incubations were performed on the first cellular passage and at 80–90% cell confluence. The incubation medium was αMEM supplemented with 10% FBS, with antibiotics. The osteoblastic phenotype was evaluated as previously reported (27). When indicated, drugs were preincubated for 30 min with the cells before pursuing experiments.

Cell viability was estimated by the percentage of lactate dehydrogenase (LDH) released (28). No significant differences after 24 h incubation were noted between untreated control hOB and cells exposed to MSUM and CPPD microcrystals up to 1 and 3 mg/ml, respectively. However, microcrystals of MSUM at 3 mg/ml were associated with 10% release of LDH from hOB. Protein content was determined by the Pierce Coomassie protein assay kit (Pierce, Rockford, IL) using BSA as a standard.

Scanning electron microscopy

At confluence, hOB were seeded on glass coverslips pretreated with poly-L-lysine (0.1% in water solution) and further incubated with MSUM or CPPD microcrystals with or without IL-1 for 24 h at 37°C. After incubation, the layered cells were vigorously washed three times and fixed with 0.5 M glutaraldehyde in phosphate buffer (0.1 M, pH 7.3) for 30 min at 4°C, rinsed twice in sucrose solution dissolved in phosphate buffer (0.1 M, pH 7.2, 310 mOsm with 6.84% sucrose) for 5 min at 4°C, and subsequently postfixed in 1% OsO_4. Dehydration was conducted through a graded series of ethanol. Specimen were then coated with 20- to 30-nm-diameter gold/palladium and examined with a JEOL JSM-840A scanning electron microscopy.

Evaluation of osteoblastic functions

The accumulation of PGE_2 was assessed by a solid phase competition enzyme immunoassay (EIA) with acetylcholinesterase (AChE) as label and a fully characterized rabbit anti-PGE_2 polyclonal Ab. AChE-labeled PGE_2 was purchased from Cayman Chemical (Ann Arbor, MI). AChE activity was measured by the colorimetric method of Ellman using an automatic reader. Evaluation of concentrations of PGE_2 was obtained by comparison of ODs recorded to those of a standard curve generated by known concentrations of synthetic PGE_2 added to the incubation medium. The detection limit was 5 pg/ml with cross-reactivities of <0.04% for 6-keto-$PGF_{1\alpha}$ and < 0.01% for leukotriene B_4, 11-dehydrothromboxane B_2, and arachidonic acid. Each sample was assessed in duplicate.

Alkaline phosphatase and osteocalcin, markers of osteoblastic activity in bone remodeling, were assessed in cells incubated in the presence of 10 nM $1,25(OH)_2D_3$ for 72 h at 37°C. Microcrystals were added for the last 24 h of incubation. Alkaline phosphatase activity in the solubilized hOB was measured spectrophotometrically by monitoring the release of *p*-nitrophenol from disodium *p*-nitrophenyl phosphate (Sigma assay kit). The activity of alkaline phosphatase was measured in milliunits per minute. The production of osteocalcin by hOB in the culture medium was measured by a specific Intact Human Osteocalcin EIA with HRP as label (Biomedical Technologies, Stoughton, MA). The release of osteocalcin was measured in nanograms per milliliter.

The production and accumulation of IL-6 and -8 were evaluated by using enzyme immunometric assays at two sites with HRP as label. Ninety-six-well plates were coated with either a monoclonal anti-IL-6 Ab (7G5/1, clone 677B6A2; BioSource International, Camarillo, CA) or a monoclonal anti-IL-8 Ab (7H4/1, clone 893A6G8; BioSource International) in phosphate buffer solution at pH 7.4. Sandwiches of Ags were made possible by using either a compatible biotinylated second monoclonal anti-IL-6 Ab (7H1/1, clone 505E23C7; BioSource International) or a compatible biotinylated second monoclonal anti-IL-8 Ab (7H1/1, clone 790A28G2; BioSource International) in phosphate buffer solution at pH 7.4 containing BSA. Ag-Ab complexes were detected by addition of a streptavidin-HRP conjugate (BioSource International) which bound to biotinylated Abs and by addition of tetramethylbenzidine as substrate of HRP. Concentrations of IL-6 and -8 were obtained from a standard curve generated by known concentrations of human rIL-6 and -8 added to the incubation medium. The detection limits were 31 and 8 pg/ml IL-6 and -8, respectively.

Analysis by immunoblot

After incubation, adhering hOB were resuspended in 150 μl of ice-cold lysis buffer (HEPES-buffered HBSS (pH 7.4), 0.5% Triton X-100, 10 μg/ml PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin); 150 μl of 2× sample buffer (1×: 62.5 mM Tris-HCl (pH 6.8), 4% SDS, 5% 2-ME, 8.5% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.025% bromophenol blue) were added; and the mixtures were boiled for 7 min. Samples were subjected to 9% SDS-PAGE and transferred to Immobilon membranes (Millipore, Bedford, MA). Equal protein loading and transfer efficiency was visualized by Ponceau red staining. The membranes were soaked for 30 min at room temperature in TBS (25 mM Tris-HCl (pH 7.6), 0.2 M NaCl, 0.15% Tween 20) containing 5% (w/v) dried milk and subsequently exposed to the anti-COX-2 rabbit polyclonal Ab (25). The membranes were washed twice in TBS and incubated with a HRP-linked donkey anti-rabbit Ab (dilution, 1/5000). Bound Abs were revealed with the ECL Western blotting detection system as specified by the manufacturer's protocol (NEN Life Science, Boston, MA).

Statistics

Although hOB were tested at confluency, some differences in cell spreading and subsequently in their synthetic capacity could occur and lead to artifactual results. To avoid such problems, concentrations of PGE_2, alkaline phosphatase, osteocalcin, IL-6, and IL-8 were corrected to the content of cellular proteins in each culture well.

Results of PGE_2, osteocalcin, IL-6 and IL-8 were expressed in nanograms per milligrams protein, and alkaline phosphatase was expressed in milliunits per minute and milligrams of protein content in each well. Values were means ± SEM and were statistically analyzed by the nonparametric Wilcoxon signed rank test. Significance was set up at 0.05.

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Results

Scanning electron microscopy of hOB in the presence of MSUM and CPPD microcrystals

The MSUM microcrystals that were observed being parallel to the hOB cell membranes were surrounded by cellular expansions. However, some MSUM microcrystals perpendicular to the osteoblastic membranes provided evidence of partial phagocytosis by hOB (Fig. 1*A*). CPPD microcrystals adhering to hOB were also partly surrounded by cellular expansions without being completely internalized (Fig. 1*B*). hOB interacting with 3 mg/ml MSUM, unlike CPPD microcrystals, exhibited several dark dots on the membrane that indicated some degree of cellular blebbing. The preincubation of hOB with IL-1 did not induce changes in the morphological aspects of hOB alone or in the presence of microcrystals (data not shown).

Effects of MSUM and CPPD microcrystals on the production of PGE_2 by hOB

The addition of MSUM or CPPD microcrystals to hOB stimulated the production of PGE_2 (Table I). Kinetic studies with both types of microcrystals at 3 mg/ml demonstrated an accumulation of PGE_2 higher than the output of unstimulated cells as early as the first hour of incubation and concentrations of PGE_2 increased with time (data not shown). However, the concentrations of PGE_2 in microcrystal-stimulated cells were statistically different from those of controls after 24 h of incubation only. Concentrations as low as 0.01 mg/ml of MSUM and CPPD microcrystals increased the production of PGE_2 by hOB. The output of PGE_2 significantly increased by 13- and 35-fold in the presence of 0.3 and 1 mg/ml MSUM microcrystals, and by 12-, 15-, and 14-fold in the presence of 0.1, 0.3, and 1 mg/ml CPPD microcrystals, respectively (Table I). Increasing the concentrations of microcrystals up to 3 mg/ml was associated with a progressive decrease of PGE_2 production. Viability of hOB in vitro was not significantly altered in the presence of 3 mg/ml CPPD microcrystals, unlike MSUM microcrystals which induced a release of 10% LDH from hOB after 24 h of incubation. Moreover, at a microcrystal concentration of 1 mg/ml, the PGE_2-stimulating activity of MSUM microcrystals was significantly stronger than that of CPPD microcrystals inducing 2 times more accumulation of PGE_2 ($p = 0.006$, $n = 11$).

Table I. *Dose response of PGE_2 produced by normal human OB in the presence of MSUM or CPPD microcrystals*[a]

	PGE_2 (ng/mg protein)	*p*
Control	1.7 ± 0.5 (18)	
MSUM (mg/ml)		
0.01	8.6 ± 6.2 (5)	NS
0.03	17.4 ± 13.0 (6)	NS
0.1	14.8 ± 9.4 (11)	NS
0.3	22.0 ± 12.4 (18)	0.01
1.0	58.9 ± 27.0 (11)	0.006
3.0	37.6 ± 24.6 (6)	NS
CPPD (mg/ml)		
0.01	13.6 ± 11.5 (5)	NS
0.03	12.3 ± 7.8 (6)	NS
0.1	21.1 ± 13.9 (6)	0.05
0.3	25.4 ± 19.9 (11)	0.05
1.0	24.3 ± 11.1 (18)	0.007
3.0	10.4 ± 5.1 (6)	NS

[a] After 24 h incubation, PGE_2 was measured in supernatants by EIA with AChE as tracer (see *Materials and Methods*). Results are expressed as means ± SEM. Statistical analysis used the Wilcoxon signed rank test; microcrystal data were compared with control data, and significance was set at 0.05. Numbers in parentheses, number of experiments tested.

Preincubation of hOB with COX inhibitors reduced the accumulation of PGE_2 generated in the presence of optimal concentrations of MSUM or CPPD microcrystals (Table II). The specific COX-2 inhibitor NS-398 abrogated the accumulation of PGE_2 induced by the addition of MSUM microcrystals, CPPD microcrystals (Table II) or IL-1 alone or in combination (data not shown) without significant change of the basal output. Dexamethasone, an inhibitor of the release of arachidonic acid, reduced the accumulation of PGE_2 in all of the conditions tested. In contrast, colchicine, which has been shown to inhibit MSUM-activated COX-2 in human monocytes (29) as well as MSUM-stimulated mobilization of calcium, production of superoxide anion and IL-1, and tyrosine phosphorylation in human neutrophils (5, 26, 28), did not decrease the generation of PGE_2 by hOB in the presence of MSUM or CPPD microcrystals. The addition of 10 mg/ml uric acid to hOB under similar experimental conditions was associated with no change of the basal output of PGE_2 (data not shown).

Synergistic stimulation of PGE_2 production by IL-1 and microcrystals

The basal production of PGE_2 by hOB was significantly increased on the addition of 1 or 10 pM IL-1 over a 24-h incubation period

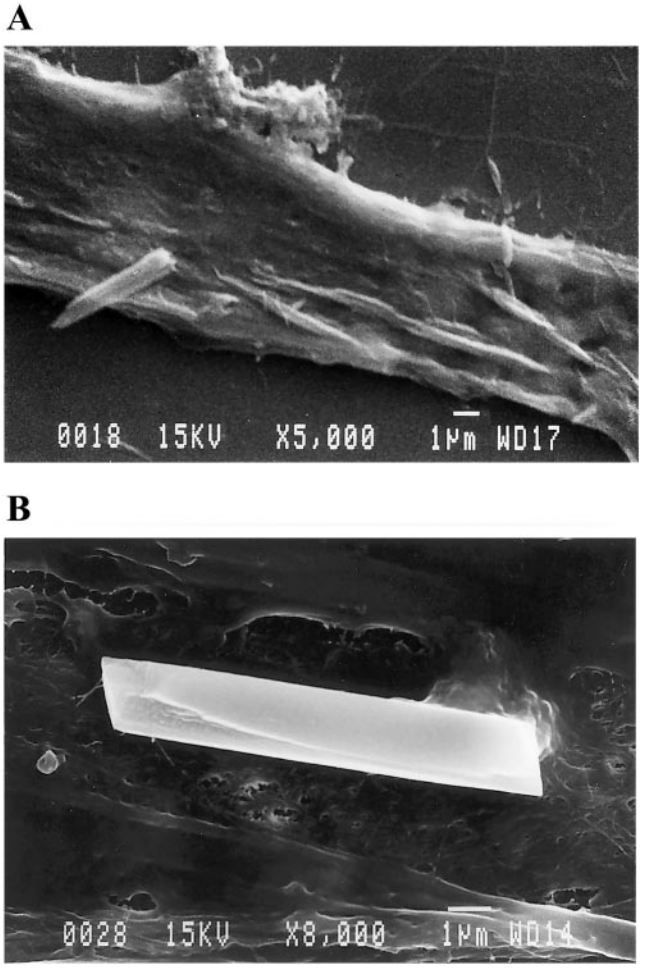

FIGURE 1. Scanning electron micrograph of human OB exposed to triclinic microcrystals of MSUM (*A*) and CPPD (*B*). Cells in direct contact with MSUM (3 mg/ml) or CPPD (3 mg/ml) are enveloping microcrystals and demonstrated morphological changes consistent with an activated state.

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Table II. *Effects of anti-inflammatory drugs on PGE_2 produced by normal human OB in the presence of MSUM or CPPD microcrystals*[a]

	Medium	MSUM (0.3 mg/ml)	MSUM (1 mg/ml)	CPPD (1 mg/ml)
Control	1.5 ± 0.5	14.9 ± 4.3	48.5 ± 20.3	3.1 ± 0.9
Indomethacin (10^{-5} M)	1.3 ± 0.5	2.2 ± 1.2*	4.2 ± 0.5*	1.2 ± 0.5*
Phenylbutazone (10^{-5} M)	1.0 ± 0.5*	1.6 ± 0.6*	5.6 ± 0.8*	1.2 ± 0.5*
Dexamethasone (10^{-6} M)	1.2 ± 0.7	2.7 ± 1.1*	2.3 ± 0.9*	1.2 ± 0.6*
Colchicine (10^{-5} M)	2.1 ± 0.9	13.5 ± 2.1	73.6 ± 33.3	5.5 ± 2.0
NS-398 (10^{-7} M)	1.2 ± 0.7	1.5 ± 0.6*	1.7 ± 0.6*	1.0 ± 0.5*

[a] Human OB were preincubated with drugs for 0.5 h at 37°C and further incubated for 24 h. PGE_2 was measured in supernatants by EIA with AChE as tracer (see *Materials and Methods*). Concentrations of PGE_2 are expressed in nanograms per milligram of protein. Results are expressed as means ± SEM. Statistical analysis (Wilcoxon signed rank test).

*, $p = 0.04$ vs control conditions ($n = 5$).

at 37°C (from 1.7 ± 0.5 to 23.7 ± 5.8 or 45.9 ± 9.7 ng/mg protein, $n = 6$, $p = 0.03$, respectively) and reached a plateau at 1 nM IL-1 (54.0 ± 12.2 ng/mg protein, $n = 18$, $p = 0.0001$). IL-1 synergistically increased the production of PGE_2 induced by MSUM and CPPD microcrystals in hOB (Figs. 2 and 3). The synergism between IL-1 and the microcrystals was time dependent, and a significant difference between IL-1-pretreated hOB alone or with microcrystals was noted after 6 h of incubation (data not shown). hOB treated with pulses of IL-1 for 1 h gave results similar to those obtained without removal of IL-1-containing medium.

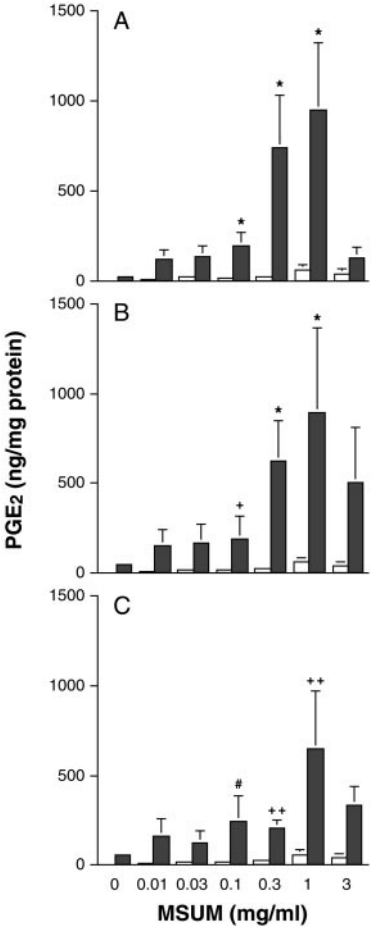

FIGURE 2. Synergism between MSUM microcrystals and IL-1 to stimulate the production of PGE_2 by human OB. Confluent hOB were pretreated with 1 pM (*A*), 10 pM (*B*), or 1 nM (*C*) IL-1 for 15 min at 37°C before adding graded concentrations of MSUM. □, MSUM alone; ■, IL-1 plus MSUM. After 24 h of incubation at 37°C, supernatants were recovered and immediately frozen at −20°C until assayed by EIA to quantitate the accumulation of PGE_2. Results are expressed in nanograms per milligram protein as means ± SEM of 6, 5, 6, 11, 18, 11, and 6 experiments at 0, 0.01, 0.03, 0.1, 0.3, 1, and 3 mg/ml MSUM, respectively. #, $p < 0.05$; +, $p = 0.04$; *, $p = 0.03$; ++, $p = 0.004$, relative to IL-1 alone.

The increases of PGE_2 accumulation induced by 0.1 mg/ml MSUM microcrystals in IL-1-pretreated hOB were significantly greater than those induced by IL-1 alone at the three concentrations of IL-1 used. Thus, 0.1 mg/ml MSUM microcrystals added to hOB pretreated with 1 pM, 10 pM, or 1 nM IL-1 increased the basal output of PGE_2 by 113-, 109-, and 141-fold, respectively (Fig. 2). The synthesis of PGE_2 induced by the three concentrations of IL-1 was also significantly increased in the presence of 0.1 mg/ml MSUM microcrystals by 8-, 4-, and 4-fold ($p = 0.03$, 0.04, and 0.05 compared with IL-1 alone, respectively, $n = 6$). The most effective combinations of IL-1 with MSUM microcrystals were recorded at 1 mg/ml MSUM microcrystals with the three concentrations of IL-1 tested. However, the strongest synergism between IL-1 and MSUM microcrystals was found at 1 pM IL-1 combined with 1 mg/ml MSUM microcrystals leading to 952 ± 368 ng PGE_2 per mg protein and corresponding to a 560-fold increase of the basal PGE_2 output and a 40-fold increase of the PGE_2 production by the IL-1-pretreated hOB (Fig. 2*A*). Increasing MSUM microcrystals concentrations up to 3 mg/ml led to a decrease of the accumulation of PGE_2 regardless of the concentrations of IL-1 used.

The addition of CPPD microcrystals to IL-1-pretreated hOB was also associated with a significant increase of PGE_2 accumulation over that of IL-1-pretreated hOB alone, and 0.03 mg/ml was the lowest concentration of CPPD microcrystals inducing a significant synergism at the three concentrations of IL-1 used (Fig. 3). Thus, 0.03 mg/ml CPPD microcrystals added to hOB pretreated with 1 pM, 10 pM, or 1 nM IL-1 increased the basal output of PGE_2 by 112-, 72-, and 98-fold (PGE_2 = 191 ± 76, 122 ± 52, and 167 ± 97 ng/mg protein compared with control, respectively, $n = 6$). The hOB synthesis of PGE_2 activated by the three concentrations of IL-1 was also significantly increased in the presence of 0.03 mg/ml CPPD microcrystals by 8-, 2.5-, and 3-fold ($p = 0.03$, 0.03, and 0.03 compared with IL-1 alone, respectively; $n = 6$). The most effective combinations of IL-1 with CPPD microcrystals were recorded at 1 mg/ml CPPD microcrystals with 1 pM or 10 pM IL-1 and 0.1 mg/ml CPPD microcrystals with 1 nM IL-1. Similarly to MSUM microcrystals, the strongest synergism was also associated with the combination of 1 mg/ml CPPD microcrystals and 1 pM IL-1 stimulating the basal production of PGE_2 by 269 times and the IL-1-activated synthesis of PGE_2 by 23 times. Although the PGE_2-stimulating activity of MSUM microcrystals in the presence

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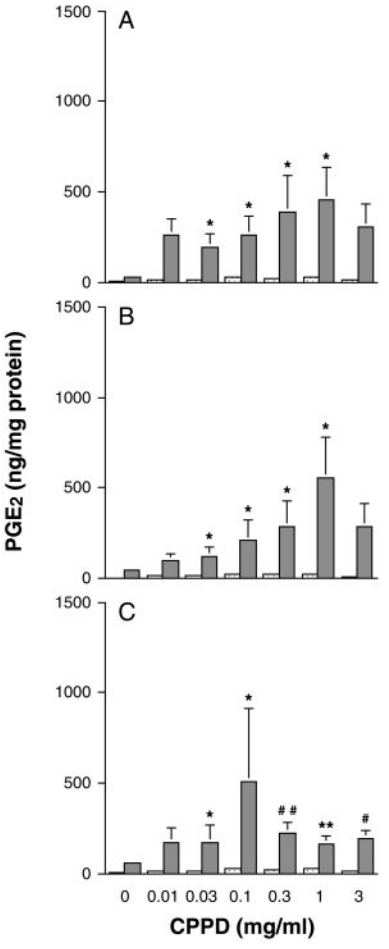

FIGURE 3. Synergism between CPPD microcrystals and IL-1 to stimulate the production of PGE_2 by human OB. Confluent hOB were pretreated with 1 pM (*A*), 10 pM (*B*), or 1 nM (*C*) IL-1 for 15 min at 37°C before adding graded concentrations of CPPD. ▨, CPPD alone; ▩, IL-1 plus CPPD. After 24 h of incubation at 37°C, supernatants were recovered and immediately frozen at −20°C until assayed by EIA to quantitate the accumulation of PGE_2. Results are expressed in ng/mg proteins as means ± SEM of 6, 5, 6, 11, 18, 11, and 6 experiments at 0, 0.01, 0.03, 0.1, 0.3, 1, and 3 mg/ml CPPD, respectively. #, $p < 0.05$; ∗, $p = 0.03$; ##, $p = 0.006$; ∗∗, $p = 0.0005$ relative to IL-1 alone.

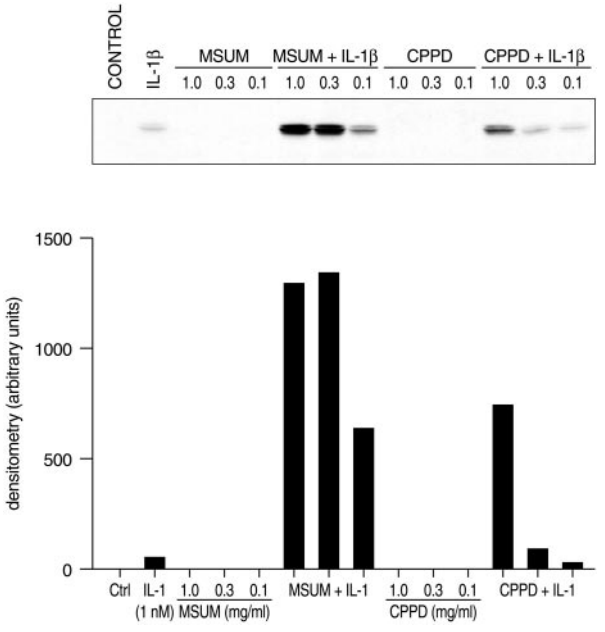

FIGURE 4. Effect of combination of MSUM or CPPD microcrystals with IL-1 on COX-2 protein expression by human OB. Confluent hOB were pretreated with 1 nM IL-1 for 15 min at 37°C before adding MSUM or CPPD (both at 1, 0.3, or 0.1 mg/ml) for 3 h at 37°C. Total proteins were prepared, and immunoblots were conducted to determine the expression of COX-2 protein. Results are from one experiment representative of three separate experiments performed on hOB from different donors.

of IL-1 was twice that of CPPD microcrystals, the difference was not statistically significant.

Enhanced expression of COX-2 induced by microcrystals and IL-1

To further investigate the effects of the microcrystals on the synthesis of PGE_2 induced in hOB in vitro, their impact on the expression of COX-2 was studied. Immunoblot studies were conducted using a rabbit polyclonal Ab against COX-2 (Fig. 4). The concentrations of microcrystals used were adapted from the data reported above. No COX-2 protein was detectable in control cultures of hOB. IL-1 at 10^{-9} M alone stimulated the appearance of a doublet with a relative molecular mass of 72 and 74 kDa, representing differentially glycosylated isoforms of the COX-2 protein as previously reported (25, 30). Overexposure of the films indicated that at 1 mg/ml, both types of microcrystals induced small but detectable increases in COX-2 expression (data not shown). The expression of the COX-2 doublet was significantly higher when IL-1-prestimulated hOB were incubated in the presence of microcrystals than in cells exposed to either agonist alone. The joint effects of the microcrystals and of IL-1 on the expression of COX-2 in hOB were clearly more than simply additive and the synergism demonstrated in the production of PGE_2 (see above) was also found in the expression of the COX-2 protein. Moreover, under similar experimental conditions, the synergism between IL-1 and MSUM microcrystals was more pronounced than that between IL-1 and CPPD microcrystals, as shown by the semiquantitative results depicted in the histogram section of Fig. 4. The increase of COX-2 protein stimulated by IL-1 and MSUM microcrystals was evident at concentrations as low as 0.1 mg/ml MSUM microcrystals. The highest quantity of immunoblotted material of COX-2 protein was recovered at 0.3 mg/ml MSUM microcrystals, and little difference was observed between the effects of 0.3 and 1 mg/ml. With CPPD microcrystals, the synergism between IL-1 and microcrystals on the expression of COX-2 protein was detected at 0.3 mg/ml CPPD microcrystals but was best observed at 1 mg/ml.

Influence of MSUM and CPPD microcrystals on the production of alkaline phosphatase and osteocalcin by hOB

MSUM microcrystals reduced by 28% ($p < 0.05$) the release of alkaline phosphatase by hOB incubated without IL-1 (Table III). The effects of CPPD microcrystals, although seemingly similar to those of MSUM microcrystals, were not statistically significant ($p = 0.13$, $n = 7$). When hOB were incubated in the presence of 1 nM IL-1, the release of alkaline phosphatase was decreased to 72% of that observed in control cells. The addition of MSUM or CPPD microcrystals to IL-1-pretreated hOB led to a significantly more pronounced, although less than additive, reduction of the release of alkaline phosphatase than in IL-1- or microcrystal-pretreated hOB alone (48 and 38% inhibition, respectively). The efficiency of MSUM microcrystals to inhibit alkaline phosphatase release was not statistically different from that of CPPD microcrystals.

The release of osteocalcin was reduced by 42% by MSUM microcrystals (Table III). The decrease of osteocalcin release induced by CPPD microcrystals was not statistically significant ($p = 0.08$,

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Table III. *Effects of MSUM and CPPD microcrystals on the release of alkaline phosphatase and osteocalcin by normal human OB*[a]

	Alkaline Phosphatase (mU/mg protein)	% of Control	Osteocalcin (ng/mg protein)	% of Control
Control	398 ± 82	100	48.0 ± 17.1	100
MSUM (0.3 mg/ml)	286 ± 73*	72	27.7 ± 8.9#	58
CPPD (1 mg/ml)	331 ± 85	83	41.3 ± 15.5	86
IL-1 (1 nM)	287 ± 67*	72	17.6 ± 10.3*	37
IL-1 + MSUM	206 ± 64*	52	12.6 ± 7.2*	26
IL-1 + CPPD	246 ± 58#	62	16.3 ± 7.2*	34

[a] Cells were incubated in the presence of 10 nM $1,25(OH)_2D_3$ for 72 h at 37°C. Vehicle or microcrystals, IL-1, or IL-1 + microcrystals were added for the last 24 h of incubation. Cells were then solubilized, the release of *p*-nitrophenol was monitored, and osteocalcin was evaluated as described in *Materials and Methods.* Results are expressed as means ± SEM. Statistical analysis (Wilcoxon signed-rank test).

*, $p = 0.02$; #, $p = 0.03$ vs control ($n = 7$).

$n = 7$). IL-1 alone significantly inhibited the release of osteocalcin to 37% of the control. The addition of MSUM or CPPD microcrystals to IL-1-preactivated hOB further reduced the release of osteocalcin. The effects of MSUM microcrystals on the inhibition of osteocalcin release were significantly greater than those of CPPD microcrystals in IL-1-treated hOB ($p = 0.03$, $n = 7$).

Influence of MSUM and CPPD microcrystals on the production of IL-6 and IL-8 by hOB

Human OB in medium alone released 37 ± 16 ng IL-6/mg protein after 24 h of incubation. MSUM and CPPD microcrystals dose-dependently stimulated the production of IL-6 by hOB. The threshold microcrystal concentration inducing a significant production of IL-6 by hOB was lower for MSUM than for CPPD microcrystals (0.03 and 0.1 mg/ml, respectively). Although 0.3 mg/ml CPPD microcrystals activated more efficiently the expression of IL-6 than 0.3 mg/ml MSUM microcrystals (increase of the production of IL-6 of control conditions by 8- and 6-fold, respectively), the difference was not statistically significant. The magnitude of IL-1-induced amplification of the basal output of IL-6 produced by hOB in our optimal conditions (14-fold with 1 nM IL-1) was in the range of that of previous studies (21). Both types of microcrystals slightly increased the production of IL-6 by hOB pretreated with 1 pM, 10 pM, or 1 nM IL-1; however, their effects were neither synergistic with nor additive to that of IL-1 (data not shown). The IL-1-stimulated expression of IL-6 by human osteoblast-like cells has previously been reported to be partly dependent on PGE_2 and COX-2 (31). When hOB were preincubated with the COX-2 inhibitor NS-398 at 10^{-7} M, we found that the production of IL-6 induced by IL-1 was reduced by 25–40% (data not shown). However, the addition of MSUM microcrystals to NS-398-pretreated hOB, unlike that of IL-1, was associated with an increase of the production of IL-6 by 1.5- to 3-fold; this increase was significant at 0.1 and 0.3 mg/ml MSUM microcrystals ($p < 0.05$, $n = 5$) as shown in Fig. 5.

Human OB incubated in control conditions generated 3 ± 1 ng IL-8/mg protein after 24 h of incubation. MSUM and CPPD microcrystals also dose-dependently stimulated the production of IL-8 by hOB. The threshold microcrystal concentration inducing the expression of IL-8 by hOB was 0.1 mg/ml for both microcrystals and the production of IL-8 was similar in the presence of MSUM or CPPD microcrystals (increases of the production of IL-8 of control conditions by 15- and 14-fold, respectively). The most effective concentration increasing the expression of IL-8 by hOB was 0.1 mg/ml for both microcrystals. The IL-1-induced increases of the basal output of IL-8 in hOB were of an order of magnitude (59 times the basal release with 10 pM IL-1) similar to those previously reported (21). MSUM and CPPD microcrystals added to IL-1-prestimulated hOB increased the formation of IL-8 induced by IL-1 alone, but the increased production was, at most, additive (data not shown). In contrast, preincubation of hOB with the COX-2 inhibitor NS-398 at 10^{-7} M did not change the production of IL-8 by hOB in the presence of IL-1 or with both types of microcrystals (data not shown). Colchicine at 10^{-5} M did not modulate significantly the production of IL-6 and -8 by hOB activated by microcrystals and/or IL-1 (data not shown).

Discussion

Inflammatory triclinic microcrystals of MSUM and CPPD, when placed in direct contact with human osteoblast-like cells in vitro, induced morphological (i.e., pseudopods surrounding microcrystals and partial engulfment of the microcrystals) and important functional changes of the hOB (i.e., expression of COX-2 and production of PGE_2, generation of cytokines such as IL-6 and -8, decrease of alkaline phosphatase activity and of osteocalcin formation). This represents the first documented evidence of the effects of inflammatory microcrystals of MSUM and CPPD on the phenotype of hOB in vitro.

Osteoblasts were previously shown capable of phagocytosis of mineralized bone particles as demonstrated by ultrastructural studies (15), of internalization of various solid particles from biomaterials with subsequent alteration of osteoblastic functions (17, 19, 32), and of phagocytosis of *Mycobacterium bovis* Calmette-Guérin bacillus with induction of bone resorption (33). The present data indicate that the activation of hOB by MSUM and CPPD microcrystals did not require their internalization by the target cells.

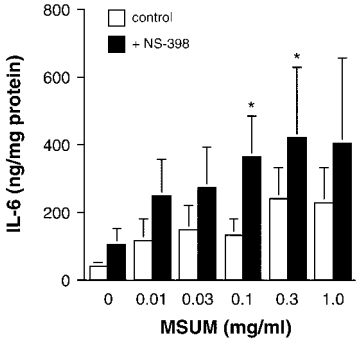

FIGURE 5. Effects of NS-398 on the production of IL-6 by human OB. Confluent hOB were preincubated with 10^{-7} M NS-398 for 30 min at 37°C before adding graded concentrations of MSUM. After 24 h of incubation at 37°C, supernatants were recovered and immediately frozen at −20°C until assayed by EIA to quantitate the accumulation of IL-6. Results are expressed in nanograms per milligram protein as means ± SEM of five experiments at all microcrystal concentrations tested; *, $p < 0.05$.

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Moreover, the functional response of hOB to MSUM and CPPD microcrystals in term of stimulation of PGE_2 and IL-6 production or in term of inhibition of $1,25(OH)_2D_3$-induced alkaline phosphatase and osteocalcin was of a similar magnitude to that previously reported with various prosthetic wear particles (17, 18). One of the mechanisms underlying the inhibition of $1,25(OH)_2D_3$-induced alkaline phosphatase and osteocalcin by inflammatory microcrystals may be the increases of endogenous PGE_2, a mechanism previously implicated in the inhibitory effects of IL-1, TNF, and parathormone (34, 35). Of relevance, MSUM, CPPD, apatite, and calcium oxalate microcrystals have been reported to stimulate the production of PGE_2 by macrophages, synovial fibroblasts, or chondrocytes in animals or humans (36–39).

Osteoblast-like cells have been identified as a major source of PGE_2 (40), the most abundant eicosanoid in bone with anabolic or catabolic effects depending on its local concentration. The present study indicated that MSUM or CPPD microcrystals were capable of stimulating hOB to express a significant activity of COX-2 at concentrations lower than those previously reported for activation of neutrophils in vitro (28, 41–45). Compared with CPPD microcrystals, the stronger activity of MSUM microcrystals to stimulate the production of PGE_2 in hOB was also demonstrated for calcium mobilization, superoxide production, and phospholipase D activity in human neutrophils (28, 41, 46), suggesting that MSUM microcrystals could be more potent activators of cellular functions than CPPD microcrystals. Also, similar to our data on aggressiveness differences between MSUM and CPPD microcrystals on hOB (Fig. 1 and LDH evaluation), particulate debris in the presence of hOB induced cytotoxicity depending on the nature of debris and their concentrations (17). The present study also indicated that MSUM or CPPD microcrystals in conjunction with IL-1β markedly overstimulated the expression of COX-2 and the accumulation of PGE_2 in hOB. Human OB were strongly stimulated by IL-1 to produce PGE_2 (47) and to express COX-2 (48). The production of PGE_2 by osteoblasts was previously reported to be synergistically stimulated by IL-1 and parathormone or TGF-β (49, 50) and by haptoglobin in the presence of bradykinin or thrombin (51). However, no data were available concerning the potential synergism between IL-1 and solid particles.

The synthesis of PGE_2 is regulated at two successive steps, the release of arachidonic acid from phospholipids through the activity of phospholipases (mainly phospholipase A_2), and its conversion to prostanoids through cyclooxygenases (COX-1 and -2) and isomerases. The expression of the inducible COX-2, a rate-limiting enzyme for the production of PGE_2, is strongly stimulated by IL-1 in hOB at the levels of mRNA and protein (48). However, IL-1 appears to be a weak activator of phospholipase A_2, and the availability of arachidonic acid is increased through the presence of serum in incubation medium and/or by addition of other agonists with strong phospholipase A_2-stimulating activity (50, 52, 53). In this connection, MSUM microcrystals are known to be potent activators of arachidonic acid release by human neutrophils and platelets (44). In the present study, inflammatory microcrystals were observed to be capable of a weak activation of the expression of COX-2 protein in hOB, an effect that was strongly enhanced in the presence of IL-1. Hence, a similar synergism was observed between the ability of the microcrystals and of IL-1 to stimulate the production of PGE_2 and the expression of COX-2 (Figs. 2–4). These results suggest that the main mechanism of the present synergism was at the level of an amplification of COX-2 protein. This interpretation is consistent with our preliminary data indicating that bradykinin, an agent known to stimulate the release of arachidonate in hOB (54), can further increase the production of PGE_2 in hOB exposed to IL-1 and to the microcrystals (data not shown). It is also important that the synergistic effects of microcrystals and IL-1 were found to occur at the level of the production of PGE_2 but not of that of the generation of IL-6 and -8 or with the modulation of $1,25(OH)_2D_3$-induced alkaline phosphatase and osteocalcin.

Previous studies have shown that hOB are capable of IL-6 production in response to proinflammatory cytokines such as IL-1 and TNF and in response to solid particles such as wear debris of various types (18, 20, 21). Inflammatory microcrystals also stimulated the generation of IL-6 by hOB as reported here. The preincubation of hOB with the COX-2 inhibitor NS-398 indicated that the IL-1-induced IL-6 formation was partly dependent on COX-2 activity as previously shown (31). In contrast, the expression of IL-6 stimulated by microcrystals was independent of COX-2 (Fig. 5), suggesting that inflammatory microcrystals can activate the osteoblastic expression of IL-6 via a PGE_2-independent pathway. The induction of IL-6 synthesis by hOB through two distinct mechanisms was also suggested by the additive effects of IL-1 and microcrystals on this production. Human OB have also been found to respond to IL-1 or TNF by producing IL-8 (21, 55), and synergistic interactions between IL-1 and TNF have been observed (56). However, the effects of solid particles and of NS-398 on the expression of IL-8 by hOB had not been previously examined. Inflammatory microcrystals stimulated the expression of IL-8 by hOB in a dose-dependent but IL-1-independent manner. This may be a reflection of a limited capacity of hOB to synthesize IL-8. Moreover, the IL-1-induced expression of IL-8 by hOB, unlike that of IL-6, was independent of the COX-2 pathway. The inflammatory microcrystals also activated the osteoblastic expression of IL-8 through a PGE_2-independent pathway.

In conclusion, our data suggest that inflammatory microcrystals profoundly alter many functions of human osteoblasts, increasing the expression of phlogogenic molecules while decreasing the bone-anabolic function of hOB. The altered activities of hOB in the presence of inflammatory microcrystals could contribute, together with other mediators, to abnormal local bone resorption associated with gout or chondrocalcinosis (pseudogout) through a reduced bone formation (decrease of alkaline phosphatase activity and osteocalcin), and an increased osteoblast-mediated osteoclastic activity (increase of PGE_2 and IL-6) with possible repercussions on bone remodeling. Inflammatory microcrystals are powerful activators of inflammatory cells such as monocytes and neutrophils, synovial fibroblasts, and endothelial cells, all cells adjacent to microcrystal foci. Thus, direct contacts between MSUM or CPPD microcrystals and bone cells may allow uncoupling of cells involved in bone remodeling.

Acknowledgments

We thank Dr. Thomas G. Kennedy for kindly donating the anti-PGE_2 Ab for EIA and Dr. Frédéric Potvin for his technical assistance.

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