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Bone Cells From Patients With Quiescent Crohn's Disease Show a Reduced Growth Potential and an Impeded Maturation

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ABSTRACT

Patients with Crohn's disease (CD) are at increased risk of developing osteoporosis. The mechanism underlying bone loss in CD patients is only partly understood. Inflammation is thought to contribute by causing a disturbed bone remodeling. In this study, we aimed to compare functional characteristics of osteoblasts from CD patients and controls, as osteoblasts are one of the effector cells in bone remodeling. The study included 18 patients with quiescent CD and 18 healthy controls. Bone cells obtained from iliac crest biopsies were cultured in the absence and presence of the inflammatory cytokines IL-1α, IL-1β, IL-6, TNF-α, IL-10, and TGF-β. At various time points, cell proliferation and differentiation were analyzed. Bone cells from CD patients showed a prolonged culture period to reach confluence and a decreased cell number at confluence. CD patient-derived bone cell cultures produced higher alkaline phosphatase levels, whereas osteocalcin levels were considerably reduced compared to control cultures. At the proliferation level, the responsiveness to inflammatory cytokines was similar in bone cells from CD patients and controls. At the differentiation level, CD cultures showed an increased responsiveness to IL-6 and a decreased responsiveness to TGF-β. Responsiveness to the other cytokines tested was unaffected. In summary, we show a reduced growth potential and impeded maturation of bone cells from quiescent CD patients in vitro. These disease-related alterations combined with an unchanged sensitivity of CD patient-derived bone cells to inflammatory cytokines, provide a new insight in the understanding of CD-associated bone loss. J. Cell. Biochem. 113: 2424–2431, 2012.

KEY WORDS: INFLAMMATORY BOWEL DISEASE; OSTEOBLASTS; PROLIFERATION; DIFFERENTIATION; CYTOKINES

Patients with inflammatory bowel disease, in particular those suffering from Crohn's disease (CD), are at increased risk of developing osteoporosis. The cause of bone loss in patients with inflammatory bowel disease is only partly understood and is thought to include malnutrition, malabsorption, calcium and vitamin D deficiency, and corticosteroid treatment [Vestergaard, 2003; Hardy and Cooper, 2009]. Accumulating evidence suggests that the inflammatory process itself plays a pivotal role in inflammatory

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bowel disease-associated bone loss as well. High local production of inflammatory cytokines in inflammatory bowel disease likely results from inappropriate and ongoing activation of the mucosal immune system [Pullman et al., 1992; Stevens et al., 1992]. Levels of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) are elevated in the circulation as well [Murch et al., 1991; Street et al., 2004]. Therefore, these cytokines might possibly mediate systemic effects of inflammatory bowel disease, such as bone loss, through the circulation.

In bone, locally derived inflammatory cytokines are pivotal in the regulation of the bone remodeling process. In order to maintain normal bone mass, bone is continuously remodeled through bone resorption by osteoclasts and bone formation by osteoblasts [Parfitt, 2002]. Inflammatory cytokines, such as TNF-α, IL-1, and IL-6, have been shown to increase osteoclast formation and osteoclast function in vitro, which might result in a stimulation of bone resorption [Manolagas and Jilka, 1995; Chambers, 2000; Takahashi et al., 2011]. On the other hand, these cytokines seem to increase osteoblast cell growth but decrease osteoblast function in several bone cell culture systems, suggestive of an inhibition of bone formation [Modrowski et al., 1995; Gilbert et al., 2000; Hughes et al., 2006]. As a consequence, the net effect of elevated pro-inflammatory cytokine levels on bone is bone loss.

In vivo studies implicate a relation between inflammation and bone loss as well. Both disease activity and serum IL-6 levels have been reported to inversely correlate with bone mineral density (BMD) and the prevalence of osteoporosis in patients with inflammatory bowel disease [Pollak et al., 1998; Paganelli et al., 2007]. Furthermore, a monoclonal antibody against TNF-α, infliximab, beneficially affects markers of bone metabolism, and increases BMD in patients with CD [Ryan et al., 2004; Bernstein et al., 2005; reviewed by Veerappan et al., 2011]. This implies that not only cytokines derived locally from bone, but also cytokines from the circulation might be involved in bone remodeling.

In a previous study, we revealed differences between bone from CD patients and bone from healthy controls by histomorphometric analysis of bone biopsies [Oostlander et al., 2011]. Prolonged exposure to a small excess of inflammatory mediators might have caused changes in bone at the tissue level, and possibly affects bone cell functionality as well. Therefore, in this study, we aimed to compare the growth potential and functional characteristics of primary human bone cells from CD patients in quiescent state of disease with cells obtained from healthy controls. Furthermore, in order to determine whether bone cells from CD patients differ from cells from healthy controls with respect to their sensitivity to inflammatory factors, we assessed the responsiveness of bone cells from quiescent CD patients and healthy controls to pro-inflammatory and anti-inflammatory cytokines on both the cell proliferation and differentiation level.

MATERIALS AND METHODS

STUDY POPULATION

Eighteen patients with quiescent CD participated in this study. These patients were a subgroup included in a large randomized, double-blind, placebo-controlled, multicenter trial on the effect of risedronate in quiescent CD patients with osteopenia ($n = 131$, Crohn and Bone study). Patients were diagnosed with CD using clinical, endoscopic, histological, and radiological criteria according to Lennard-Jones [1989]. Patients were in remission (C-reactive protein [CRP] levels <10 mg/L, and Crohn's Disease Activity Index [CDAI] <150), and had a lumbar spine and/or total hip BMD with a T-score of -1 to -2.5 SD (osteopenia). Patients with current or recent bisphosphonate or corticosteroid treatment (respectively <1 year and <3 months prior to inclusion) were excluded as well as patients with metabolic bone diseases and/or vitamin D deficiency (serum 25-hydroxyvitamin D levels <25 nmol/L). Disease-specific medication not belonging to the exclusion criteria, that is, immunosuppressive drugs, was used by 8 out of 18 patients.

From nine male and nine female CD patients aged 40.4 ± 10.7 years (mean $\pm$ SEM), transiliac bone biopsies were obtained at baseline ($n = 5$) or after 2 years of placebo or risedronate treatment ($n = 7$ and $n = 6$, respectively). From patients with two biopsies available, only the biopsy obtained at baseline was used. None of the female CD patients was post-menopausal. Controls included healthy donors who underwent a pre-implant bony reconstruction of the maxilla and/or mandible, using an autologous bone graft from the anterior iliac crest. During the surgical procedure, superfluous trabecular bone fragments were collected. The control group consisted of 10 male and 8 female subjects aged 41.0 ± 15.2 years (mean $\pm$ SEM). Three of eight female controls were post-menopausal. The study was approved by the Institutional Review Board of the VU University Medical Center, and all subjects gave written informed consent.

CELL CULTURE

Cell culture was performed using a modification of earlier described methods [Klein-Nulend et al., 2002]. Briefly, part of the trabecular bone of transiliac biopsies was dissected within 1–4 h after removal, minced into small fragments, washed repeatedly with PBS, and incubated with 2 mg/ml collagenase (type II; Worthington, NJ) for 2 h at 37°C in a shaking water bath. After washing with medium containing 10% Fetal Clone I (HyClone), the bone fragments were subdivided into portions and transferred to 25 cm^2 culture flasks. Culture flasks were weighed before and after addition of bone fragments to determine wet weight. Wet weight of the bone portions was 71 ± 7 mg (mean $\pm$ SEM) in healthy controls and 60 ± 5 mg in patients with CD. Therefore, the bone fragment density in CD cultures was similar to that of healthy controls. Culture flasks were maintained at 37°C in a humidified atmosphere with 5% CO_2. The culture medium consisted of DMEM/F-12 (Dulbecco's Modified Eagle's Medium: nutrient mixture F-12; Gibco) with 10% Fetal Clone I, 100 U/ml penicillin and 100 μg/ml streptomycin mixture (Gibco), and 1.25 μg/ml fungizone (Gibco), and was changed twice a week. The bone phenotype of the cultured cells was confirmed by staining several control cultures for the presence of alkaline phosphatase activity (ALP staining) and calcium phosphate deposition (von Kossa staining) (data not shown).

CELL PROLIFERATION

Primary human bone cells at passage one were used to examine cell proliferation. Cells were seeded into 96-well culture plates (Greiner

Bio-One, Frickenhausen, Germany) at a density of 2.5×10^3 or 5×10^3 cells per well in culture medium without phenol-red, and allowed to attach overnight. The following day, vehicle (PBS), recombinant human IL-1α (10 ng/ml), IL-1β (10 ng/ml), IL-6 (10 ng/ml) plus its soluble receptor (IL-6sR; 100 ng/ml), IL-10 (1 ng/ml), TGF-β (10 ng/ml), or TNF-α (10 ng/ml) was added. Cell proliferation was determined after 3, 5, and 7 days of culture using the Cell Proliferation Kit II (XTT; Roche) according to the manufacturer's instructions. Metabolically active cells convert the XTT reagent into a colored solution, which correlates to the number of proliferating cells. Absorbance at 450 nm was measured using an automated plate reader (BioTek Instruments, Winooski, VT). Experiments were performed in triplicate and repeated independently using material from 8 to 10 different donors.

CELL DIFFERENTIATION

Primary human bone cells at passage two were used for differentiation experiments. Cells were seeded into 6-well culture plates (Costar, Corning, Inc.) at a density of 2.5×10^4 cells per well and grown to 50–60% confluence. Medium was changed to osteogenic medium consisting of the standard medium plus 50 μg/ml ascorbic acid (Sigma) and 10 mM β-glycerophosphate (Sigma), and treatments were added. Treatment consisted of the addition of vehicle (PBS, recombinant human IL-1α (1 ng/ml), IL-1β (1 ng/ml), IL-6 (1 ng/ml) plus its soluble receptor (IL-6sR; 100 ng/ml), IL-10 (100 pg/ml), TGF-β (1 ng/ml), or TNF-α (1 ng/ml). Media and treatments were replenished twice a week. Three days before measurements, 10 nM vitamin D_3 (Sigma) and 10 nM vitamin K_1 (Sigma) were added to the cell cultures to facilitate bone cell maturation. After 1, 2, 4, and 6 weeks of culture, medium was collected to determine collagen and calcium production, cell lysates were made to measure alkaline phosphatase activity, and RNA was isolated to determine the expression of osteoblast differentiation markers. Experiments were repeated independently using material from three to four different donors.

P1NP synthesis. To ascertain collagen production by the bone cells, procollagen type 1 amino-terminal propeptide (P1NP) concentration in conditioned medium was assessed using the UniQ P1NP radioimmunoassay kit from Orion Diagnostica. Measurements were performed according to manufacturer's instructions. Samples were stored at −20°C after collection until all samples of one experiment could be analyzed at once.

Calcium synthesis. To ascertain calcium production by the bone cells, calcium concentration in conditioned medium was assessed using a colorimetric assay from Roche. Measurements were performed according to manufacturer's instructions. Samples were stored at −20°C after collection until all samples of one experiment could be analyzed at once.

Alkaline phosphatase activity. To quantify alkaline phosphatase (ALP) activity in cell lysates, cells were washed in PBS, lysed in ice-cold water, harvested by scraping, sonicated (2×30 s at 50/60 Hz), and centrifuged at 5,000 rpm for 5 min. Supernatants were collected and analyzed using the ALP IFCC liquid assay (Roche), based on the method described by Lowry [Lowry et al., 1954] and according to manufacturer's instructions. After measurement samples were stored at −20°C, Alkaline phosphatase activity was normalized to total protein content, which was quantified in all samples of one experiment at once using the BioRad protein assay (BioRad).

RNA isolation and real-time RT-PCR. Total RNA was isolated from cells using the RNeasy® Mini kit with an on-column DNase I digestion (Qiagen, Basel, Switzerland), and stored at −80°C prior to use for RT-PCR analysis. Total RNA concentration was measured with the Nanodrop spectrophotometer (Nanodrop Technologies). One hundred nanogram of RNA was reverse-transcribed using 10 ng/μl random primers (Roche) and 5 U/μl M-MLV Reverse Transcriptase (Promega) in a mixture containing 5 mM $MgCl_2$, 1× RT-buffer, 1 mM dNTPs each, 1 M betaine and 0.40 U/μl RNAsin. In a total volume of 20 μl, the samples were incubated for 10 min at 25°C, 1 h at 37°C and 5 min at 95°C. Three μl of cDNA was amplified by PCR using the following primer sets: bone specific alkaline phosphatase (ALP; Eurogentec) forward: 5′-CCACgTCTTCACATTTggTg-3′, reverse: 5′-gCAgTgAAgggCTTCTTgTC-3′; Collagen1α1 (Col1α1; Eurogentec) forward: 5′-gTgCTAAAggTgCCAATggT-3′, reverse: 5′-ACCAggTTCACCgCTgTTAC-3′; Osteocalcin (OC; Invitrogen) forward: 5′-ggCgCTACCTgTATCAATgg-3′, reverse: 5′-TCAgCCAACTCgTCA-CAgTC-3′; and the housekeeping gene TATA-box binding protein (TB; Invitrogen) forward: 5′-ggTCTgggAAAATggTgTgC-3′, reverse: 5′-gCTggAAAACCCAACTTCTg-3′. The PCR reaction was performed on the iCycler system (BioRad) and consisted of an initial denaturation step for 3 min at 95°C, followed by 40 amplification cycles (15 s at 95°C, 1 min at 60°C) in a total volume of 25 μl containing 300 nM primers and SYBR® Green Supermix (BioRad). Following PCR, a melting curve was run to check the specificity of the reaction. Data were analyzed with the iCycler IQTM software, and compared using the ΔΔCt method.

STATISTICAL ANALYSIS

Data are expressed as mean ± standard error of the mean (SEM). Results are presented as treatment-over-control ratios (T/C ratios). Culture characteristics of bone cells from healthy controls and CD patients as well as from CD patients either or not using immunosuppressives were compared using a two-tailed Student's *t*-test. Differences between cultures from baseline, follow-up placebo-treated and follow-up risedronate-treated CD patients were tested by univariate ANOVA and Bonferroni's post hoc test. Data from control and experimental cell cultures were compared using univariate ANOVA and Dunnett's post hoc test. All analyses were performed using SPSS for Windows version 16.0. A *P*-value of <0.05 was considered statistically significant.

RESULTS

CELL CULTURE

Cell outgrowth from the bone fragments was visible after 1–4 days of culture for both fragments obtained from healthy controls and from patients with CD. At confluence, control cultures contained elongated cells showing parallel alignment (Fig. 1A). CD cultures hardly reached confluence, however, at their maximal cell density CD cultures frequently showed a less organized cell pattern with more polygonal-shaped cells (Fig. 1B). Control cultures reached confluence after 28 ± 2 days, whereas cultures from CD patients reached maximal cell density after 47 ± 3 days ($P < 0.0001$; Fig. 1C).

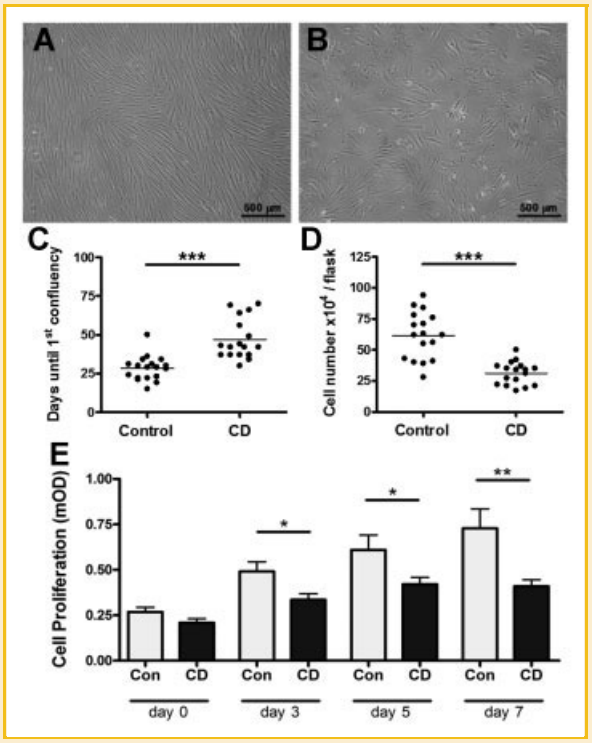

Fig. 1. Reduced cell growth and decreased cell number of primary human bone cells from patients with CD in comparison to bone cells from healthy controls. A: Cell number and morphology of primary human bone cells from healthy controls at confluence (P0); magnification 40×. B: Cell number and morphology of primary human bone cells from patients with CD at their maximal cell density (P0); magnification 40×. C: Time to reach maximal cell density was considerably higher in bone cell cultures from CD patients than in cultures from healthy controls. D: Cell number at first passage was remarkably lower in CD cell cultures compared to healthy control cell cultures. E: Basal cell proliferation (in the absence of inflammatory cytokines) was lower in bone cell cultures from CD patients than in cultures from healthy controls. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

The cell number at first passage was $(62 \pm 5) \times 10^4$ cells/culture flask for control cultures, but only $(31 \pm 5) \times 10^4$ cells/culture flask for CD cultures ($P<0.0001$; Fig. 1D). The differences in cell growth and cell number were independent of the wet weight of the bone portions as well as gender, age and BMD of the subjects. Moreover, cell growth and cell number were not significantly different between cultures from CD patients with and without using immunosuppressive drugs.

CELL PROLIFERATION

Cell density did not influence the proliferative response of bone cells to inflammatory cytokines, since no statistically significant difference in cell proliferation was observed between cytokine-treated cultures seeded at 2.5×10^3 and 5×10^3 cells/well. Therefore, analysis was performed on pooled data obtained from cultures at both cell densities. Bone cells from CD patients were obtained from both patients at baseline and follow-up (either placebo or risedronate-treated). Statistical analysis showed no difference in cell proliferation between cultures obtained from baseline, follow-up placebo-treated and follow-up risedronate-treated patients. Thus, data were pooled for further statistical analysis.

Basal cell proliferation was statistically significant lower in cultures from CD patients compared to cultures from healthy controls (Fig. 1E) and was most strongly reduced in patients treated with immunosuppressive drugs ($P<0.001$ vs. healthy controls as well as vs. CD patients not using immunosuppressive drugs; data not shown). The proliferative response of bone cells to the tested inflammatory cytokines was similar in cultures from healthy controls and CD patients, and was independent of the use of immunosuppressive drugs. IL-1α increased the proliferation of primary human bone cells in a time-dependent manner compared to untreated cell cultures (Fig. 2A). Cell proliferation increased by 58% in healthy controls and by 69% in patients with CD. Addition of IL-1β to primary human bone cell cultures resulted in an increase of cell proliferation (Fig. 2B), which was time-dependent in healthy control cultures, with a maximum increase of 48%. In cultures from CD patients the increase of proliferation was constant over time, with a maximum increase of 39%. IL-6 slightly increased cell proliferation over time in healthy control cultures, although this effect did not reach statistical significance (Fig. 2C). Proliferation of CD cultures was also not affected by IL-6. IL-10 increased proliferation of bone cells from healthy controls by 79% and

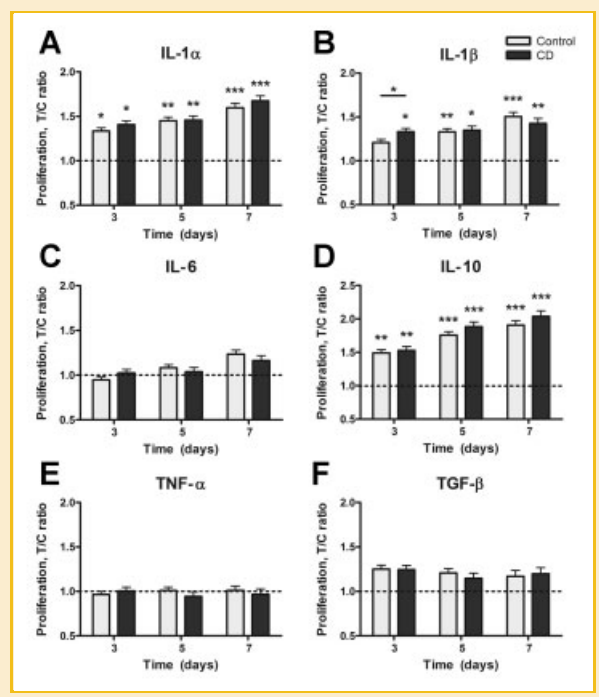

Fig. 2. The effect of pro-inflammatory and anti-inflammatory cytokines on the proliferation of primary human bone cells from healthy controls and from patients with CD. Cells were cultured in the presence of vehicle or the inflammatory cytokines IL-1α (10 ng/ml), IL-1β (10 ng/ml), IL-6 (10 ng/ml), IL-10 (1 ng/ml), TGF-β (10 ng/ml), or TNF-α (10 ng/ml) for 3, 5, and 7 days. Data represent cytokine-treated-over-control (T/C) ratios: proliferation of the vehicle condition of each study population and of each measured time point separately was set at 1.0 (dashed line; T/C = 1 is no effect). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to vehicle-treated cells, and $^{\#}P<0.05$ compared to control cultures.

proliferation of bone cells from CD patients by 104% (Fig. 2D). TGF-β and TNF-α did not affect the proliferation of primary human bone cells from either healthy controls or patients with CD (Fig. 2E,F).

CELL DIFFERENTIATION

Levels of differentiation markers showed no statistically significant difference between cultures obtained from baseline, follow-up placebo-treated and follow-up risedronate-treated CD patients. Therefore, data were pooled for further statistics.

The basal phenotype of bone cells from healthy controls and CD patients was compared based on RNA expression and protein levels of several osteoblast differentiation markers (Table I). All differentiation markers showed a relatively constant level over time, therefore, data of all time points were analyzed together as well. Due to the limited number of experiments, no distinction could be made between the bone phenotype of cultures from CD patients with and without using immunosuppressive drugs. Collagen levels were comparable between bone cell cultures from healthy controls and CD patients (RNA $P = 0.401$; protein $P = 0.638$). Alkaline phosphatase RNA levels were 30% higher in CD cultures when compared to healthy control cultures ($P = 0.001$), and alkaline phosphatase activity increased by 20% in CD cultures ($P = 0.001$). Osteocalcin RNA levels were decreased by 72% in CD cultures in comparison to healthy control cultures ($P < 0.001$). Calcium levels were up to 40% lower in CD cultures when compared to healthy control cultures ($P = 0.028$).

At the differentiation level, the responsiveness of bone cells from healthy controls and CD patients to inflammatory cytokines could not be evaluated for each time point separately due to insufficient statistical power. Analysis of data from all time points together revealed an in general comparable response to the tested inflammatory cytokines in healthy control cultures and CD cultures (Fig. 3). Details are described below.

Collagen 1α1 expression (Fig. 3A) was decreased by IL-1α in both healthy control cultures and CD cultures to respectively 41% and 35% of vehicle-treated cultures (both $P < 0.001$). IL-10 decreased collagen 1α1 expression to 65% of vehicle-treated cultures only in cultures from healthy controls ($P = 0.004$). TNF-α tended to decrease collagen 1α1 expression in CD cultures ($P = 0.052$). As healthy control cultures did not respond to TNF-α, nor to IL-6, the responsiveness of bone cells to these cytokines differed significantly between healthy control cultures and CD cultures ($P = 0.003$ and $P = 0.002$, respectively). Collagen (P1NP) production (Fig. 3B) was decreased by IL-1α in both healthy control cultures and CD cultures to respectively 67% and 46% of vehicle-treated cultures ($P < 0.001$ and $P = 0.002$, respectively).

Alkaline phosphatase expression (Fig. 3C) and activity (Fig. 3D) were significantly increased by IL-1α only in healthy control cultures to respectively 203% and 139% of vehicle-treated cultures ($P = 0.015$ and $P = 0.005$, respectively). Since alkaline phosphatase expression and activity tended to increase in CD cultures as well, the responsiveness to IL-1α was not statistically significant different between control and CD cultures. IL-6 increased alkaline phosphatase activity in cultures from CD patients to 132% of vehicle-treated cultures ($P = 0.022$). As healthy control cultures did not respond to IL-6, the responsiveness of bone cells to this cytokine differed significantly between healthy control cultures and CD cultures ($P = 0.035$). TGF-β decreased alkaline phosphatase activity only in cultures from healthy controls to 66% of vehicle-treated cultures ($P = 0.008$).

Osteocalcin expression (Fig. 3E) was decreased by IL-1α in both healthy control cultures and CD cultures to respectively 32% and 27% of vehicle-treated cultures ($P = 0.006$ and $P = 0.031$,

TABLE I. Basal Phenotype of Bone Cells In Vitro Derived From RA Patients and Healthy Individuals

	Time (weeks)				
	1	2	4	6	Total[a]
Rel. Col1α1 expression					
Control	925 ± 60 (100)	1110 ± 98 (120)	697 ± 62 (75)	750 ± 102 (81)	870 ± 43 (94)
CD	1485 ± 99 (160)[b]	1015 ± 86 (110)	630 ± 104 (68)	516 ± 100 (56)	934 ± 63 (101)
P1NP level (μg/mg protein)					
Control	3.93 ± 0.22 (100)	4.07 ± 0.39 (104)	2.78 ± 0.24 (71)	2.70 ± 0.27 (69)	3.39 ± 0.15 (86)
CD	4.81 ± 0.37 (122)	4.44 ± 0.39 (113)	2.93 ± 0.38 (75)	1.84 ± 0.31 (47)	3.52 ± 0.21 (90)
Rel. ALP expression					
Control	13.65 ± 1.38 (100)	15.85 ± 1.48 (116)	15.24 ± 2.38 (112)	14.39 ± 1.85 (105)	14.80 ± 0.91 (108)
CD	20.55 ± 1.75 (151)[c]	17.54 ± 1.19 (128)	21.34 ± 2.43 (156)	16.72 ± 2.16 (122)	19.17 ± 0.97 (140)[b]
ALP activity (μmol/mg protein/min)					
Control	0.80 ± 0.06 (100)	1.02 ± 0.06 (128)	0.93 ± 0.09 (116)	0.91 ± 0.10 (114)	0.91 ± 0.04 (114)
CD	0.91 ± 0.09 (114)	1.06 ± 0.08 (133)	1.28 ± 0.07 (160)[c]	1.14 ± 0.08 (143)	1.10 ± 0.04 (138)[b]
Rel. OC expression					
Control	7.09 ± 1.16 (100)	5.56 ± 0.74 (78)	5.67 ± 1.36 (80)	5.26 ± 1.32 (74)	5.90 ± 0.58 (83)
CD	1.89 ± 0.33 (27)[b]	1.56 ± 0.42 (22)[b]	1.05 ± 0.18 (15)[d]	2.16 ± 0.52 (31)	1.65 ± 0.19 (23)[b]
Calcium level (μmol/mg protein)					
Control	NA	NA	8.66 ± 1.18	8.31 ± 1.22	8.51 ± 0.84
CD	NA	NA	7.48 ± 1.58	5.29 ± 0.64[d]	6.04 ± 0.69[d]

NA, not applicable.
Data are expressed as mean ± SEM; in parenthesis, data are shown relative to healthy control (week 1).
[a]Total: pooled data from all time points tested.
[b]Statistically significant different from the control group ($P < 0.001$).
[c]Statistically significant different from the control group ($P < 0.01$).
[d]Statistically significant different from the control group ($P < 0.05$).

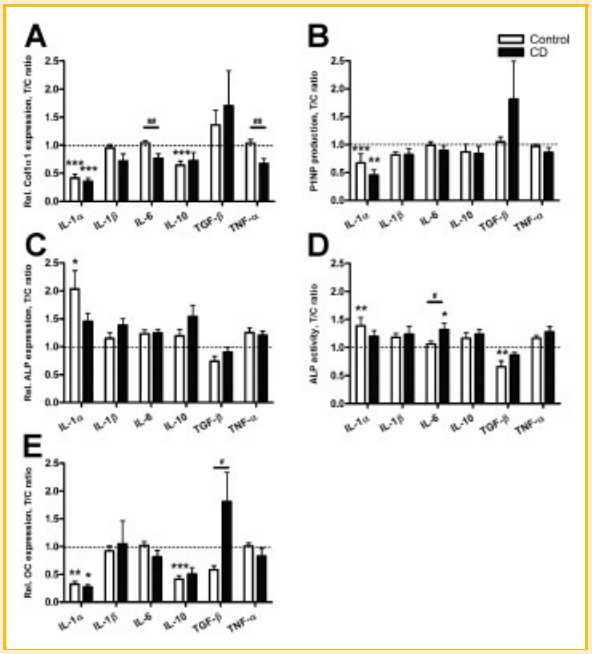

Fig. 3. The effect of pro-inflammatory and anti-inflammatory cytokines on the differentiation of primary human bone cells from healthy controls and from patients with CD. Cells were cultured in the presence of vehicle or the inflammatory cytokines IL-1α (1 ng/ml), IL-1β (1 ng/ml), IL-6 (1 ng/ml), IL-10 (100 pg/ml), TNF-α (1 ng/ml), or TGF-β (1 ng/ml). Data represent cytokine-treated-over-control (T/C) ratios: differentiation of the vehicle condition of each study population individually was set at 1.0 (dashed line; T/C = 1 is no effect). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to vehicle-treated cells, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to control cultures.

respectively). IL-10 decreased osteocalcin expression in healthy control cultures by 42% of vehicle-treated cultures ($P < 0.001$) and caused a tendency towards a decrease in CD cultures ($P = 0.058$). In addition, only in healthy control cultures TGF-β decreased osteocalcin expression to 58% of vehicle-treated cultures ($P = 0.002$). As CD cultures did not respond to TGF-β, the responsiveness of bone cells to this cytokine differed significantly between healthy control cultures and CD cultures ($P = 0.020$).

DISCUSSION

In the present study, we demonstrated that the growth potential of primary human bone cells obtained from patients with quiescent CD is diminished in comparison to that of bone cells from healthy controls. A reduced growth rate and a decreased cell number at maximal cell density were observed in bone cell cultures from CD patients. BMD did not correlate with the changes in growth rate and cell number. Changes in bone cell density of the bone fragments are also not likely to have caused the observed differences, as we showed in a previous study that osteocyte density within bone biopsies is comparable between controls and CD patients [Oostlander et al., 2011]. Cell growth and viability are not only related to (initial) cell density but to cell morphology as well [Price, 1997]. In the present study, cultured healthy control bone cells had an elongated cell shape and showed parallel alignment at confluence. CD bone cell cultures showed a less organized cell pattern with more polygonal-shaped cells at their maximal cell density. Therefore, the decreased growth potential of cells from our CD patients might also be related to a change in cell morphology. In this study, no data on cell survival are available. Whether increased apoptosis in CD patient-derived bone cells has accounted for the observed decrease in cell growth and cell number as well as for the observed changes in cell organization level remains to be elucidated. Nonetheless, taken together, our findings suggest a phenotypic alteration in osteoblasts of patients with CD.

A second interesting finding is an impeded maturation of primary human bone cells obtained from patients with CD when compared to that of bone cells from healthy controls. Alkaline phosphatase RNA and protein levels were elevated in bone cells from CD patients, whereas osteocalcin RNA levels and calcium excretion were considerably reduced in cells from CD patients. Osteocalcin and calcium are produced by well-differentiated osteoblasts. The downregulation of these proteins in cultures of CD patients might imply that bone cells of patients with CD stagnate in their development before reaching a fully mature phenotype. The differences in expression of osteoblast differentiation markers in bone cells from CD patients again pinpoint towards a functional change in this cell. Studies in the field of immunology and oncology, have increased the awareness of the role of microenvironmental factors, including persistent subclinical inflammation, in the determination of a cell's phenotype [Vakkila and Lotze, 2004; Mosser and Edwards, 2008; Postovit et al., 2008]. Therefore, we propose that the observed alterations in both cell proliferation and function of bone cells from CD patients in vitro might reflect disease-specific modifications caused by chronic subclinical inflammation or other CD-related factors in vivo.

Since phenotypic differences between bone cells from CD patients and healthy controls might affect their responsiveness to inflammatory cytokines, we studied the effect of several pro-inflammatory and anti-inflammatory cytokines on the proliferation and differentiation of primary human bone cells from both healthy controls and patients with CD. In general, the response to the tested inflammatory cytokines was comparable between cultures from healthy controls and cultures from CD patients. However, bone cells from CD patients showed an enhanced responsiveness to IL-6 during the early to intermediate phase of differentiation, as reflected by reduced collagen expression and elevated alkaline phosphatase activity in comparison to IL-6 treated healthy control bone cell cultures. The increased sensitivity for IL-6 in CD bone cells suggests a pivotal role for IL-6 in the pathogenesis of CD-associated bone loss, especially when considering elevated serum IL-6 levels commonly observed in CD patients with active disease. CD patient-derived bone cells seemed to have a decreased responsiveness to TGF-β during the intermediate to late phase of differentiation, as reflected by unaffected differentiation markers in comparison to decreased alkaline phosphatase and osteocalcin levels in cultures from healthy controls. However, it must be mentioned that CD cultures had strongly reduced basal levels of osteocalcin, which might have hampered the detection of a decrease of this late phase differentiation marker in bone cells from CD patients. All taken together, these

findings indicate that bone cells from CD patients remain susceptible to elevated levels of circulating inflammatory cytokines. As such, our results fit in with the current view on the importance of inflammation in CD-associated bone loss.

One of the strengths of this study is the use of a primary human bone cell culture system. Previous studies have shown that human primary cultures are more representative of the in vivo osteoblast and therefore more suitable to study the mechanism of (inflammation-induced) bone loss than the commonly used animal or cell line culture systems [Harbour et al., 2000]. A disadvantage of the use of primary human bone cells is the relatively mature state of the cells, which impaired us to study the early phase of osteoblast differentiation. However, the early phase of osteoblast differentiation has been studied by Bernardo et al. [2009] who showed that mesenchymal stem cells of CD patients have similar characteristics, including their osteogenic potential, to cells of healthy donors.

In this study, for the first time isolated bone cells from patients with quiescent CD were studied. Characteristics of bone cells from CD patients were directly compared with bone cells from healthy controls, thereby excluding bias from methodological variations. A drawback of this study might be the pooling of CD patients treated with a bisphosphonate or placebo. However, proliferation as well as differentiation of the bone cells was comparable between bisphosphonate-treated and placebo-treated patients, and therefore bisphosphonate treatment is not likely to have influenced the outcome of this study. On the other hand, bone cells from patients treated with immunosuppressive drugs showed a greater reduction in cell proliferation than bone cells of patients not receiving any treatment. Data on a direct effect of methotrexate and azathioprine (the immunosuppressive drugs used by the patients included in this study) on bone cells in vitro is limited and not very consistent [Scheven et al., 1995; Uehara et al., 2001; Minaur et al., 2002; Malviya et al., 2009]. Therefore, the more pronounced reduction in cell proliferation in bone cells of patients on immunosuppressive therapy might be the result of an indirect effect of these drugs. Possibly, a relatively more active state of disease in patients treated with immunosuppressive drugs in comparison to patients not receiving any treatment has contributed to our findings.

The experiments performed in this study required fresh bone samples form patients and controls, which are evidently hard to obtain especially from patients already suffering from chronic intestinal disease. A consequent limitation of this study is the relatively small sample size and restricted experimental setup. Nonetheless, all results obtained consistently point towards a functional change in osteoblasts of patients with CD. This knowledge may contribute to further experimental and clinical research.

In conclusion, we demonstrated a reduction in the growth potential of primary human bone cells obtained from patients with quiescent CD in comparison to bone cells obtained from healthy controls. In addition, we showed an impeded maturation of CD patient-derived bone cells. The overall responsiveness of bone cells from CD patients to inflammatory cytokines, known to be involved in active disease, remained largely unaffected. Therefore, the disease-specific, phenotypic alterations combined with the unchanged sensitivity of CD patient-derived bone cells to inflammatory cytokines, provide a new insight in the understanding of bone loss frequently observed in patients with CD.

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