Vitamin B$_{12}$ Deficiency Stimulates Osteoclastogenesis via Increased Homocysteine and Methylmalonic Acid

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Abstract The risk of nutrient deficiencies increases with age in our modern Western society, and vitamin B$_{12}$ deficiency is especially prevalent in the elderly and causes increased homocysteine (Hcy) and methylmalonic acid (MMA) levels. These three factors have been recognized as risk factors for reduced bone mineral density and increased fracture risk, though mechanistic evidence is still lacking. In the present study, we investigated the influence of B$_{12}$, Hcy, and MMA on differentiation and activity of bone cells. B$_{12}$ deficiency did not affect the onset of osteoblast differentiation, maturation, matrix mineralization, or adipocyte differentiation from human mesenchymal stem cells (hMSCs). B$_{12}$ deficiency caused an increase in the secretion of Hcy and MMA into the culture medium by osteoblasts, but Hcy and MMA appeared to have no effect on hMSC osteoblast differentiation. We further studied the effect of B$_{12}$, Hcy, and MMA on the formation of multinucleated tartrate-resistant acid phosphatase–positive osteoclasts from mouse bone marrow. We observed that B$_{12}$ did not show an effect on osteoclastogenesis. However, Hcy as well as MMA were found to induce osteoclastogenesis in a dose-dependent manner. On the basis of these results, we conclude that B$_{12}$ deficiency may lead to decreased bone mass by increased osteoclast formation due to increased MMA and Hcy levels.

Keywords Nutrition · Vitamin B$_{12}$ · Homocysteine · Methylmalonic acid · Osteoporosis

Osteoporosis is a complex multifactorial disease with a strong genetic component, but lifestyle factors are also important for maintenance of bone mass. Nutrition is one of the factors that contributes to bone health. During the first decades of life, adequate intake of calcium and vitamin D is essential to establish peak bone mass (see [1, 2] and references therein). It has also been observed that nutritional deficiencies can cause impaired growth and skeletal abnormalities during childhood [3], while in adults inadequate intake of nutrients such as proteins, vitamins, and minerals has been associated with loss of bone quality [2, 4]. Nutritional deficiency is very common in the elderly, emphasizing that adequate nutrient intake may slow down the age-related loss of bone mass.

A challenge in nutrition research is to identify food components that contribute to the maintenance of bone health. During the last years, vitamin B$_{12}$ has gained particular attention since a number of epidemiological studies have reported a correlation between B$_{12}$ status and bone quality and fracture risk [5–9]. The micronutrient B$_{12}$ is involved as a cofactor in two enzymatic reactions [10]. The enzyme methionine synthase requires B$_{12}$ for the remethylation of homocysteine (Hcy) into methionine. The other enzyme dependent on B$_{12}$ is methylmalonyl-CoA mutase, which catalyzes the conversion of methylmalonyl-CoA into succinyl-CoA. As a consequence of B$_{12}$ deficiency, these

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enzymatic reactions are impaired and Hcy and methylmalonic acid (MMA) accumulate [11].

In the elderly, B$_{12}$ uptake is reduced and the prevalence of low B$_{12}$ levels as well as moderate hyperhomocysteinemia and elevated serum MMA levels increases with age [12]. Several studies have reported that low plasma B$_{12}$ levels are associated with reduced bone quality [5, 8, 9, 13, 14], but this relation has not been observed by others [15–17]. More consistently, however, are the observations that increased Hcy may be a predictive factor for fracture risk [13–15, 18–21]. Also, elevated MMA levels have been associated with osteoporosis in the elderly [14] and low bone mineral density (BMD) in adolescents [6]. Together, these data suggest that B$_{12}$, Hcy, and MMA are important factors in the development of osteoporosis.

The mechanisms by which B$_{12}$ deficiency leads to impaired bone quality are still poorly understood. A number of studies have described the effects of B$_{12}$ and Hcy on osteoblasts and osteoclasts [22–27]. Here, we analyzed the effects of B$_{12}$, Hcy, and MMA on human mesenchymal stem cells (hMSCs), the precursor cells of both osteoblasts and adipocytes. In theory, a number of mechanisms may underlie the reduced bone quality in cases of B$_{12}$ deficiency. B$_{12}$ may have a stimulatory effect on bone formation via enhanced osteoblast differentiation or improved bone matrix mineralization. B$_{12}$ may also act in a negative way on osteoclast formation and function. It can be hypothesized that Hcy and MMA may act in an opposite manner, i.e., reduced osteoblast formation and increased osteoclast formation. In the present study we examined the effects of varying levels of B$_{12}$, Hcy, and MMA on the differentiation of MSCs into osteoblasts as well as on osteoclastogenesis from bone marrow cultures.

Materials and Methods

Cell Culture

hMSCs were obtained from Lonza (Verviers, Belgium; male donors, lot numbers 6F3502 and 6F4392) and cultured in proliferation medium as supplied by the manufacturer. Murine MC3T3-E1 osteoblast precursor cell, obtained from the American Type Culture Collection (Manassas, VA), were maintained in α-MEM supplemented with 10% fetal bovine serum (FBS, Lonza), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO$_2$. For differentiation experiments, cells were seeded at a density of $1.0 \times 10^4/\text{cm}^2$ in proliferation medium. Two days after cell seeding, the medium was replaced with osteogenic or adipogenic differentiation medium. Osteogenic medium for hMSCs consisted of DMEM (Lonza) supplemented with 10% FBS, penicillin/streptomycin, L-glutamine, 100 nM dexamethasone, 50 μg/ml ascorbic acid, 10 nM vitamin D, and 12.5 mM β-glycerophosphate. Osteogenic medium for MC3T3-E1 cells consisted of DMEM/10% FBS supplemented with penicillin/streptomycin, L-glutamine, 50 μg/ml ascorbic acid, and 12.5 mM β-glycerophosphate.

The DMEM medium did not contain B$_{12}$ according to the manufacturer's specification sheet. Determination of B$_{12}$ levels (Lab SHO, Velp, The Netherlands) in DMEM was below the detection limit of 40 pM, while FBS contained 125 pM B$_{12}$.

Cells were cultured in osteogenic medium (B$_{12}$-deficient) or osteogenic medium supplemented with B$_{12}$ mimicking physiological levels (500 pM) or high-B$_{12}$ supplementation (50 nM). Adipogenic differentiation was stimulated by culturing cells in DMEM/10% FBS supplemented with penicillin/streptomycin, L-glutamine, 1 μM dexamethasone, 1 μM rosiglitazone, 0.45 mM isobutylmethylxanthine, and 5 μg/ml insulin. During differentiation, the medium was refreshed twice a week.

DL-Homocysteine, B$_{12}$ (cyanocobalamin), and MMA were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Alkaline Phosphatase Assays

Alkaline phosphatase (ALP) cytochemical staining was performed using the Alkaline Phosphatase Sigma Diagnostics kit 85, according to the protocol supplied by the manufacturer. ALP activity was determined by measuring the formation of *p*-nitrophenol from *p*-nitrophenyl phosphate (PNPP, Sigma-Aldrich). Briefly, cells were cultured in 96-well plates, washed twice with PBS, and fixed in 4% paraformaldehyde/PBS for 10 minutes at 4°C. Subsequently, cells were incubated with PNPP for 15 minutes at 37°C, after which the reaction was terminated with NaOH and the absorbance of *p*-nitrophenol was measured at 414 nm. ALP activity was corrected for cell number as determined by a neutral red assay. Cells were incubated with neutral red dye diluted in PBS for 1 hour at 37°C. After washing with PBS, the dye was extracted from the cells with 0.05 M NaH$_2$PO$_4$ in 50% EtOH and absorbance was measured at 540 nm. The results of the neutral red assay revealed that cell numbers were not changed by the different culture conditions. Measurements were performed sixfold and averaged.

Mineralization Assays

Calcium concentrations were determined in a colorimetric assay with *o*-cresolphthalein complexone as a chromogenic agent. Cells were washed twice with PBS, after which

calcium was extracted with 0.5 M HCl by incubating overnight with continuous shaking.

Calcium was quantified by mixing 50 µl of sample with 950 µl of assay solution containing calcium binding reagent (0.024wt% o-cresolphthalein and 0.25wt% 8-hydroxyquinone in H$_2$O) and calcium buffer (500 mM 2-amino-2-methyl-1,3 propanediol in H$_2$O) in a 1:1 volume mixture. The absorbance of each solution was measured at 575 nm. All reagents were obtained from Sigma-Aldrich.

Triglyceride Analysis

For quantification of lipid accumulation during adipogenesis, cells were grown in 24-well plates. Cells were washed twice with PBS, after which lipids were extracted by shaking for 10 minutes in 50 µl of 25 mM Tris–HCl (pH7.5)/1 mM EDTA, 40 µl of tert-butanol, and 10 µl of methanol. After drying the plates, triglycerides were determined spectrophotometrically using the triglyceride Liquicolor Monoreagent (Instruchemie, Delfzijl, The Netherlands). Triglyceride formation was expressed as fold changes averaged over biological duplicate measurements.

The accumulation of lipid droplets during adipogenic differentiation was visualized by staining with oil red O (ORO) in combination with ALP staining as described above. After staining for ALP, cells were washed twice with water and incubated with ORO staining solution containing two volumes of 50 mg ORO in 10 ml isopropanol and three volumes of water. After staining for 10 minutes at 37°C, cells were washed twice with water and pictures were taken.

RNA Isolation and Real-Time PCR

Total RNA was extracted from cells using TRIzol® Reagent (Invitrogen, Breda, The Netherlands), and RNA concentrations were determined by measuring the absorbance at 260 nm.

Total RNA (1 µg) was reverse-transcribed with the use of the cDNA synthesis kit (Promega, Leiden, the Netherlands). Subsequently, cDNA was amplified in a quantitative real-time PCR performed with the use of Platinum® Taq DNA polymerase (Invitrogen) and SYBR® green on an iCycler PCR machine (Bio-Rad, Veenendaal, The Netherlands). The data were normalized by measuring threshold cycle ratios between the candidate genes and a housekeeping gene, *RPS27A* (ribosomal protein S27a). The following primer pairs were used: *RPS27A* (forward), GTTAAGCTGGCTGTCCTGAAA, *RPS27A* (reverse), CATCAGAAGGGCACTCTCG; *ALP* (forward), GATGGACAAGTTCCCCTTCGT, *ALP* (reverse), GGACCTGGGCATTGGTGTT; *COL1A1* (forward), GTCGAGGGCCAAGACGAAG, *COL1A1* (reverse), CAGATCACGTCATCGCACAAC; *IBSP* (forward), GCAAAATTAAAGCAGTCTTCATTTTG, *IBSP* (reverse), TGCCTTGAGCCTGCTTCC; *OPG* (forward), AAGGGCGCTACCTTGAGATAG, *OPG* (reverse), GCAAACTGTATTTCGCTCTGGG.

Osteocalcin Assay

Osteocalcin in cultured medium form murine MC3T3-E1 cells was determined by an immunoradiometric assay (Immutopics, San Clemente, CA). The detection limit is 29 ng/ml, intra-assay variation is 4.55%, and interassay variation is 6.1%.

Determination of Hcy and MMA Levels

Total Hcy levels secreted by hMSCs and MC3T3 cells were determined in the cell culture medium by an automated HPLC method with reverse-phase separation and fluorescent detection as described earlier [28]. MMA concentrations in the culture medium were measured with the use of a liquid chromatography mass spectrometry method (LC-MS-MS).

Osteoclastogenesis

Osteoclast differentiation from bone marrow cultures was performed as described previously [29]. Whole bone marrow isolated from femurs of male C56/BL6 mice was cultured in 96-well plates at a density of 1.0×10^5 cells/well on plastic and on 650-µm-thick bovine cortical long bone slices. Cells on plastic were cultured in 150 µl of DMEM/5% FBS medium containing 30 ng/ml recombinant murine M-CSF (R&D Systems, Minneapolis, MN) and 20 ng/ml RANKL (Biovision, Mountain View, CA) to induce osteoclast formation. Cells on bone slices were cultured in 150 µl α-MEM/5% FCS supplemented with 30 ng/ml M-CSF and 20 ng/ml recombinant mouse RANKL (R&D Systems). Cells were cultured for 7 or 9 days in the presence of B$_{12}$, Hcy, or MMA on plastic or for 6 days on bone slices. The medium was refreshed after 3 and 6 days. RANKL (Biovision) was kindly provided by Dr. R. Arends (Organon, Oss, The Netherlands).

Tartrate-Resistant Acid Phosphatase Assay

Osteoclast cultures were washed with PBS, fixed, and stained for tartrate-resistant acid phosphatase (TRAP) using the Sigma-Aldrich Acid Phosphatase Kit 387, according to the protocol supplied by the manufacturer. After staining, TRAP-positive cells with three or more nuclei were counted and pictures were taken. Student's t-tests (two-tailed) were carried out to compare values

between two groups. Differences between groups were considered significant at $P < 0.05$ (two-tailed).

Results

Vitamin B$_{12}$ Deficiency Does Not Affect Osteoblast Differentiation

To examine the effect of B$_{12}$ deficiency on MSC differentiation, bone marrow–derived hMSCs were used since these cells have been described as multipotent cells which efficiently differentiate toward the osteoblast and adipocyte lineage [30, 31]. We first investigated the effect of B$_{12}$ on the onset of osteoblast differentiation as characterized by expression of ALP [32]. To exclude the possibility that substantial baseline levels of B$_{12}$ were present in the culture medium, a quantitative measurement of B$_{12}$ levels was carried out. Baseline levels were lower than 15 pM, which is far below the B$_{12}$ deficiency level of 200 pM [13]. Culturing hMSCs in osteogenic differentiation medium resulted in a homogeneous population of ALP-positive cells after 9 days of culture, as visualized by cytochemical staining for ALP (Fig. 1a). Supplementation with 50 nM B$_{12}$ had no effect on osteoblast formation from hMSCs when characterized by cytochemical staining. An identical result was observed with physiological concentration of 500 pM (not shown). Similarly, a quantitative assay revealed a strong induction of ALP activity in osteogenic medium compared to control medium, but no effect of B$_{12}$ on ALP activity was detected (Fig. 1b), even when cells were treated with concentrations up to 5 μM (not shown). ALP activity was not changed by B$_{12}$ by repeating the experiment with cells obtained from another donor (not shown).

To study the effect of B$_{12}$ on an alternative differentiation pathway of hMSCs, these cells were cultured in adipogenic medium and triglyceride content was determined after 7 and 9 days of differentiation (Fig. 1c). A strong increase in triglyceride formation was observed in adipogenic medium compared to control medium, but the triglyceride content was not significantly changed when B$_{12}$ was supplied to the medium. These data show that the onset of hMSC differentiation toward osteoblasts or adipocytes is not influenced by B$_{12}$ deficiency.

We further examined the effect of B$_{12}$ on osteoblast maturation by measuring calcium deposition in the extracellular matrix after 7, 9, and 11 days of differentiation. The data presented in Fig. 1d show that hMSCs strongly accumulated calcium in the matrix under osteogenic conditions. B$_{12}$ supplementation of 0.5 or 50 nM had no effect on calcium levels, a result that was verified by means of a von Kossa staining (not shown), indicating that B$_{12}$ has no effect on matrix mineralization.

To analyze B$_{12}$ effects in more detail, osteoblast gene expression was determined by means of quantitative real-time PCR (Q-PCR). Expression of the osteoblast marker genes *ALP*, *COL1A1* (collagen type 1 alpha 1), *IBSP* (integrin-binding sialoprotein or bone sialoprotein), and *OPG* (osteoprotegerin) was determined in cells cultured for 2, 4, 7, and 9 days in control medium, osteogenic medium, or osteogenic medium supplemented with 50 nM B$_{12}$. Figure 1e shows that the mRNA levels of these genes increased in osteogenic medium but that gene expression was not modulated by B$_{12}$ supply.

Taken together, these results show that B$_{12}$ has no effect on osteoblast differentiation of hMSCs. To exclude a cell type–specific effect, the murine preosteoblast cell line MC3T3-E1 was tested for its response to B$_{12}$. Figure 1f shows that ALP activity was induced in osteogenic medium after 4 days but, as in hMSCs, no effect of B$_{12}$ could be detected, indicating that B$_{12}$ had no effect on the early phase of MC3T3-E1 differentiation. As a marker for osteoblast maturation, secreted osteocalcin levels were determined in the medium of differentiating MC3T3 cells. As can be seen in Fig. 1g, osteocalcin secretion was strongly increased in osteogenic medium compared with control medium during a 3-week culturing period. However, no changes in osteocalcin secretion were detected under the influence of different B$_{12}$ levels.

Vitamin B$_{12}$ Deficiency Increases Hcy and MMA Secretion by Osteoblasts

To investigate whether B$_{12}$ deficiency correlates with Hcy and MMA secretion by osteoblasts, total Hcy (tHcy) and MMA secreted in the culture medium were measured during osteoblast differentiation of hMSCs and MC3T3-E1 cells (Fig. 2). As can be seen in Fig. 2a, tHcy secreted by hMSCs in osteogenic medium increased during a differentiation period of 10 days. After 10 days of differentiation, secreted tHcy levels were reduced by 20% when the cells were cultured in the presence of 50 nM B$_{12}$. This result shows that lower B$_{12}$ levels result in higher tHcy secretion by osteoblasts. As in hMSCs, lowering B$_{12}$ levels caused increased tHcy secretion by MC3T3-E1 (Fig. 2b). During a 20-day period of differentiation, a dose-dependent increase in tHcy secretion was observed when cultures were treated with 50, 0.5, or 0 nM B$_{12}$.

Secretion of MMA in the culture medium of hMSCs appeared to be unchanged by varying B$_{12}$ levels during a differentiation period of 10 days (Fig. 2c). In MC3T3-E1 cells, however, secreted MMA levels showed a dose-dependent response to B$_{12}$ (Fig. 2d). MMA secretion was attenuated in the presence of 0.5 and 50 nM B$_{12}$, and the effect increased during the progress of differentiation. The observed differences between supplementation of 0 and
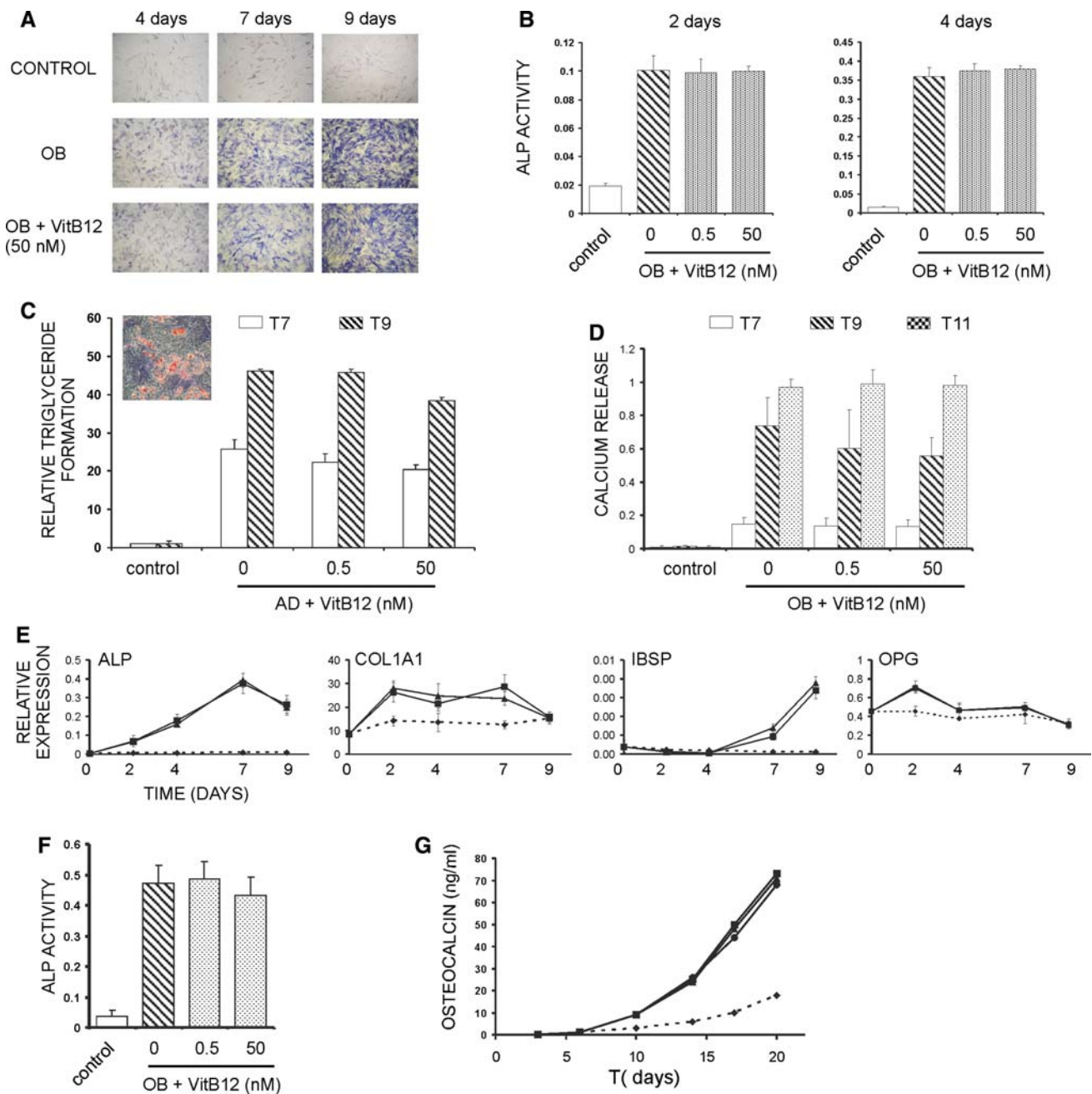
Fig. 1 The effect of B$_{12}$ on MSC differentiation. **a** Cytochemical analysis of ALP expression in hMSCs cultured in control medium, in osteogenic differentiation medium (OB), or in OB supplemented with 50 nM B$_{12}$ for 4, 7, and 9 days. **b** Quantification of ALP expression in hMSCs cultured in OB medium supplemented with B$_{12}$. Measurements were performed sixfold, averaged, and normalized for cell numbers by a neutral red assay. Error bars indicate standard deviation. **c** Triglyceride formation by hMSCs was quantified after 7 (*white bars*) and 9 (*dashed bars*) days of differentiation in control medium, adipogenic differentiation (AD) medium, or AD medium supplemented with B$_{12}$. Triglyceride formation is represented as fold change relative to control at t7 and t9. Adipocyte formation at t7 was indicated by the accumulation of lipid droplets that stain with oil red O (*inset*). **d** Calcium release by hMSCs after 7, 9, or 11 days of

culture in OB medium supplemented with 0, 0.5, or 50 nM B$_{12}$. Calcium was determined in a colorimetric reaction and quantified by measuring the absorbance at 575 nm. **e** Gene expression levels of osteoblast markers *ALP*, *COL1A1*, *IBSP*, and *OPG* in hMSCs cultured over a period of 9 days in control medium (◆), OB medium (■), or OB medium supplemented with 50 nM B$_{12}$ (▲). Measurements were performed in duplicate, after which relative expression was calculated by correction for expression of the housekeeping gene *RPS27A*. **f** ALP activity of murine MC3T3 osteoblast precursor cells in control medium, OB medium, or OB supplemented with B$_{12}$ over a period of 4 days. **g** Osteocalcin secretion in culture medium by MC3T3 cells cultured for a period of 20 days in control medium (◆), OB medium (■), or OB medium supplemented with 0.5 nM B$_{12}$ (●) or 50 nM B$_{12}$ (▲)

Fig. 2 The effect of B_{12} on Hcy and MMA secretion by osteoblasts. tHcy and MMA levels were determined during differentiation of hMSCs (**a, c**) and MC3T3 cells (**b, d**) for a period of 10 and 20 days, respectively. tHcy and MMA were determined at the indicated time points and are shown as cumulative values. Cells were cultured in OB medium (♦), or OB medium supplemented with 0.5 nM B_{12} (■) or 50 nM B_{12} (▲)
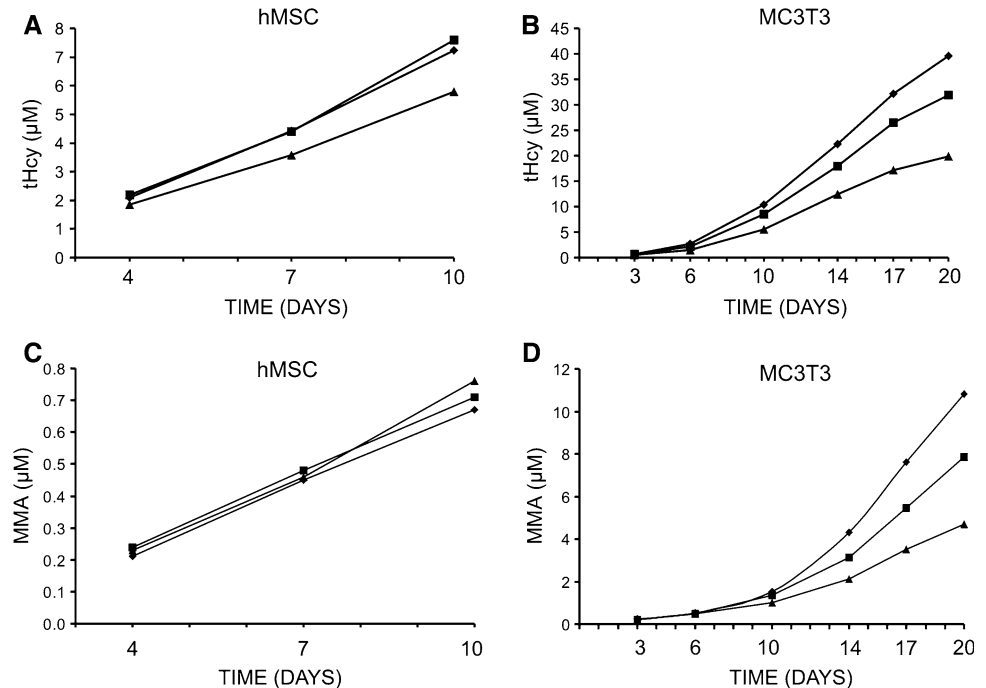
0.5 nM B_{12} indicate that prolonged B_{12} deficiency results in increased Hcy and MMA secretion by osteoblasts.

Hcy and MMA Do Not Reduce Osteoblast Differentiation

To analyze the effect of Hcy on the onset of osteoblast differentiation, ALP activity was determined in hMSCs cultured in osteogenic medium in the presence of different concentrations of Hcy. Figure 3a shows that Hcy concentration up to 500 μM had no effect on ALP activity after 4 days of differentiation. Also, no effect was found at other time points (data not shown). We further investigated the effect of Hcy on mineral deposition by differentiating hMSCs. Quantification of the amount of calcium released from the extracellular matrix revealed no difference due to the presence of an increasing amount of Hcy (Fig. 3b), and an identical result was obtained by von Kossa staining (not shown).

To study the effect of MMA levels on osteoblast differentiation, ALP activity was analyzed in hMSCs after 4 days of differentiation (Fig. 3c). Similar to the results obtained with Hcy, no effect of MMA on ALP activity was observed, indicating that Hcy and MMA do not modulate osteoblast differentiation of MSCs.

Hcy and MMA Stimulate Osteoclastogenesis

We next examined the effects of B_{12}, Hcy, and MMA on the formation of osteoclasts from whole bone marrow cultures. To characterize the effect of these molecules on

osteoclast formation, multinucleated TRAP-positive cells were counted and shown as average number of osteoclasts per well. B_{12} was found to have no effect on the formation of multinucleated TRAP-positive cells (Fig. 4a). However, supplementation with Hcy resulted in a dose-dependent increase in osteoclast formation (Fig. 4a). A low dose of 5 μM Hcy already led to a 2.2-fold increase in the average number of multinucleated osteoclasts per well. The number of osteoclasts further increased with higher concentrations of Hcy, showing a significant increase ($P < 0.05$) at 50 μM Hcy (Fig. 4a). A very strong increase was observed in the presence of 500 μM Hcy (Fig. 4a, b). Similar to Hcy, an increase in osteoclast numbers was observed when cells were cultured in the presence of MMA (Fig. 4c). These data therefore show that both Hcy and MMA are able to increase osteoclast formation. To analyze whether these effects of Hcy and MMA could be observed on osteoclast formation in a more physiological situation, bone marrow cells were cultured on bone slices. As can be seen in Fig. 4d, a dose of 200 μM Hcy stimulated osteoclast formation. While MMA was able to increase osteoclast formation on plastic, the effect on bone slices was not significant at a concentration of 200 μM MMA.

We finally examined whether B_{12} supplementation affects osteoclastogenesis in case of increased Hcy (Fig. 4e). A dose of 100 μM Hcy caused a significant induction of osteoclast formation, which was not seen when 50 nM B_{12} was added. Thus, while under standard culture conditions B_{12} had no effect on osteoclast formation, B_{12} was able to reduce the Hcy-induced osteoclastogenesis.
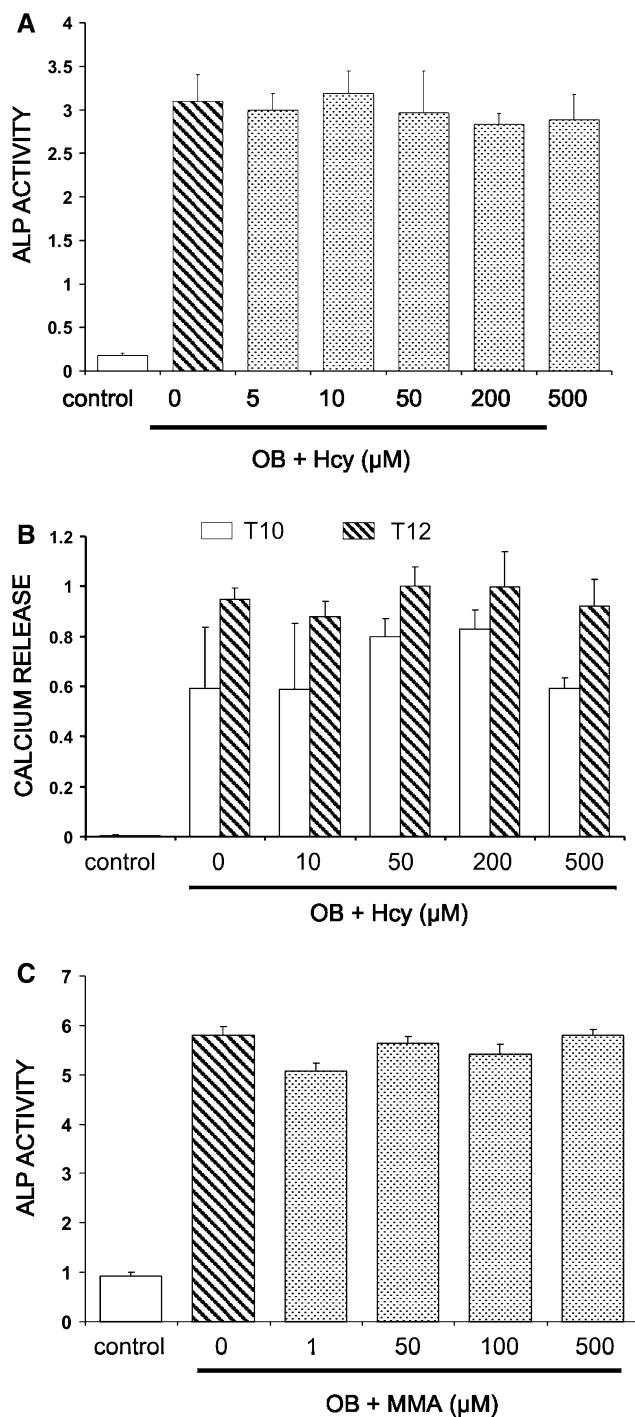
Fig. 3 The effect of Hcy and MMA on osteoblast differentiation. Quantification of ALP activity in hMSCs in OB medium supplemented with Hcy (**a**) or MMA (**c**) after 4 days of differentiation. Measurements were performed sixfold, averaged, and normalized for cell numbers by a neutral red assay. **b** Calcium release assay by hMSCs after 10 days (*white bars*) and 12 days (*hatched bars*). Cells were cultured in OB medium in the presence of Hcy 0–500 μM. Calcium was determined in a colorimetric reaction and quantified by measuring the absorbance at 575 nm. Error bars indicate standard deviation (*n* = 4)

Discussion

We and others have previously shown that B$_{12}$ deficiency and increased Hcy and MMA levels are associated with impaired bone health in the elderly [6, 8, 9, 14, 21]. In order to elucidate the molecular mechanism behind these clinical observations, we now examined the effects of B$_{12}$, Hcy, and MMA on the differentiation of bone marrow cell cultures.

Our study is the first that has analyzed the effects of B$_{12}$, Hcy, and MMA on MSC differentiation. A number of previous studies have appeared with contradictory results with respect to the effect of B$_{12}$ on other osteoblast model systems. A stimulating effect on ALP expression was reported in human rib–derived osteoprogenitors [25], rat osteosarcoma cells [25], and chicken calvaria–derived osteoblasts [22]. More recently, it was shown that simultaneous reduction of B$_{12}$, B$_6$, and folic acid levels had no influence on ALP expression in human osteoblasts [24].

Since MSCs are a more relevant model system to cover all stages of osteoblast differentiation, we extensively studied the effect of B$_{12}$ on these cells. Furthermore, we used mouse MC3T3 osteoblasts to study the effect of B$_{12}$ on cells which are at a later stage of osteoblast differentiation. We found that B$_{12}$ (1) did not affect the onset of osteoblast differentiation, (2) did not alter the stem cell switch, (3) did not affect matrix protein formation, and (4) did not affect matrix calcification. However, B$_{12}$ significantly reduced Hcy and MMA secretion by differentiating osteoblasts. Importantly, increased Hcy and MMA had no effect on osteoblast differentiation or function, indicating that B$_{12}$ deficiency neither directly nor indirectly alters bone formation by osteoblasts.

Based on these results, it is reasonable to expect that the B$_{12}$-associated bone loss as observed in clinical studies is mediated by osteoclasts. Indeed, we have found that elevated levels of Hcy as well as MMA had a stimulatory effect on the formation of multinucleated TRAP-positive osteoclasts. In contrast, various levels of B$_{12}$ did not affect the formation of osteoclasts. Gene expression analysis in osteoblasts revealed that *OPG* expression was not affected by B$_{12}$, while expression of *RANKL* could not be detected in hMSCs (data not shown). Our data are therefore in agreement with a study in which no relation was found between Hcy levels and OPG and RANKL in peri- and postmenopausal women [33]. As OPG and RANKL are known to regulate osteoclastogenesis, these results imply that B$_{12}$ deficiency does not indirectly affect osteoclast formation via RANKL and OPG secretion. Together, our data suggest a mechanism by which B$_{12}$ deficiency leads to increased Hcy and MMA levels in the bone microenvironment, thereby stimulating the formation of osteoclasts.
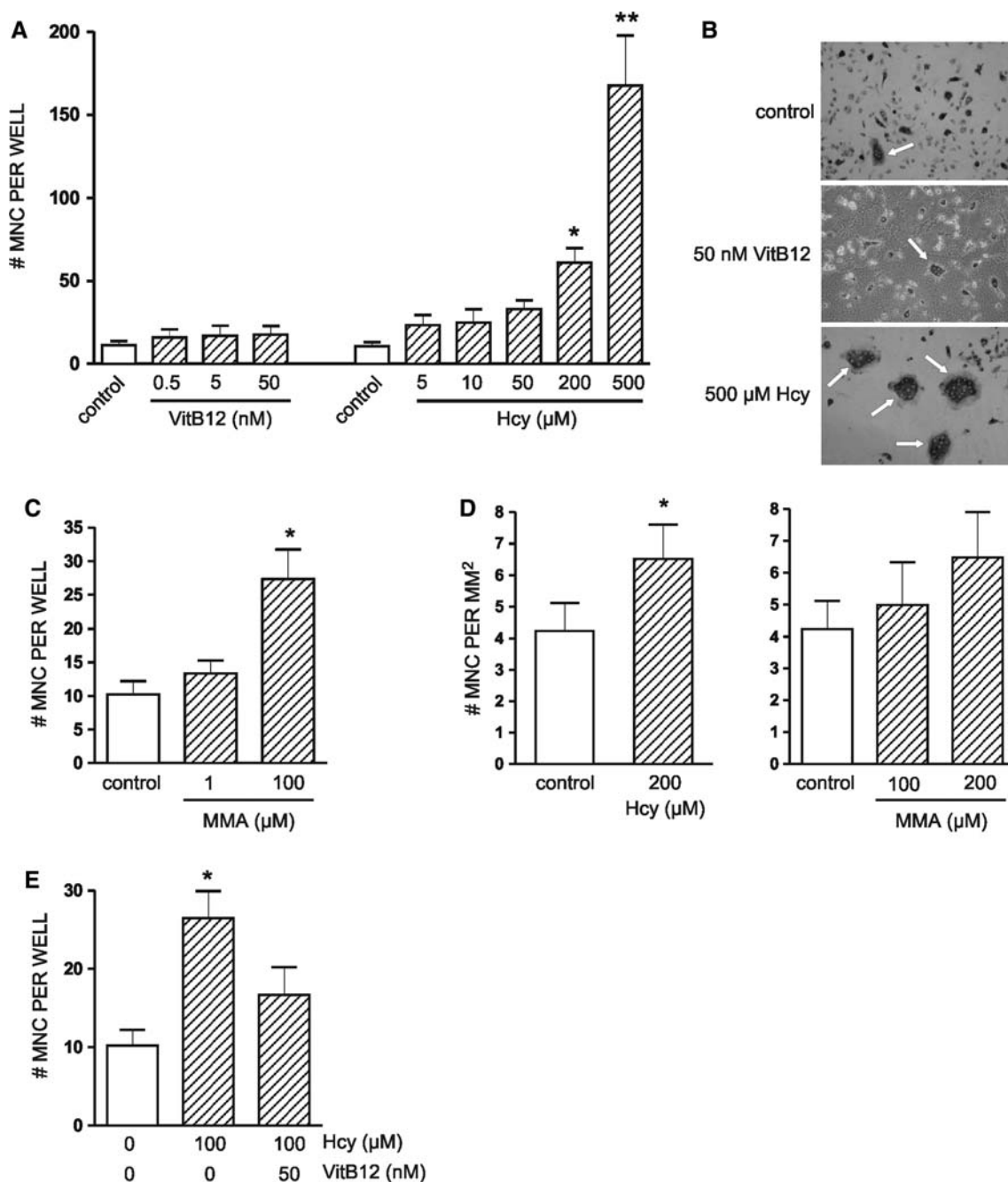
Fig. 4 The effects of B$_{12}$, Hcy, and MMA on osteoclast formation. Mouse bone marrow cells cultured with M-CSF and RANKL were treated with different concentrations of B$_{12}$, Hcy, and MMA. After TRAP staining, TRAP-positive multinucleated ($n > 2$) cells (MNCs) were counted. **a** Number of MNCs per well in 96-well plates after 7 days of culture ($n = 6$ mice, average number of MNCs). Hcy showed a significant induction of MNCs at 50 μM (*$P < 0.05$, **$P < 0.005$) and higher concentrations. **b** Formation of MNCs in the presence of high B$_{12}$ and Hcy concentration. *Arrows* indicate TRAP-positive MNCs. Note that at 500 μM Hcy the number of nuclei per cell is strongly increased. **c** Number of MNCs per well in the presence of MMA ($n = 6$ mice, each measurement performed in duplicate, 9 days of culture). **d** Osteoclasts per millimeter squared on bone slices ($n = 5$ mice, each measurement performed in duplicate, 6 days of culture). **e** Reduction of Hcy-induced osteoclast formation by B$_{12}$ ($n = 6$ mice, 9 days of culture). Error bars indicate standard error of the mean

The effect of MMA on osteoclastogenesis has not been described to date. The stimulatory effect of low concentrations of Hcy on osteoclast formation is in agreement with a study by Koh and colleagues [26]. These data provide in vitro support for our previous observations that low B$_{12}$ levels (<200 pM) and increased Hcy (>15 μM) in women result in elevation of urinary secreted deoxypyridinoline levels, which is a marker for collagen degradation,

a measure for bone resorption [13]. Our data indicate that not only Hcy but also MMA may contribute to bone loss in case of B_{12} deficiency.

Our study implies that, with respect to clinical applications, B_{12} may reduce osteoclast formation in case of high Hcy concentrations and that reduction of Hcy and MMA levels may be a promising strategy to prevent the increased fracture risk in the elderly. Animal studies in mice and rats have already revealed that modulating Hcy levels by means of methionine-enriched diets caused an increase in bone resorption [34, 35], indicating that nutrient intervention can modulate bone resorption via alteration of Hcy levels. In the elderly, Hcy levels can effectively be reduced by means of B_{12} and folate supplementation [36–38]. The novel observation that MMA is also able to induce osteoclast formation indicates that not only Hcy but also MMA levels need to be attenuated in order to prevent osteoporosis via nutrient supplementation. It has previously been shown that B_{12} supplementation leads to a reduction in plasma MMA levels in elderly with B_{12} deficiency [36, 39]. To date, studies on the effect of B_{12} and/or folate supplementation on bone health are limited. BMD and bone formation markers were found to increase in a group of osteoporotic patients after 6 months of B_{12} and calcium supplementation [40]. A larger study in patients after stroke showed that Hcy levels, fracture risk, and BMD were reduced after treatment with B_{12} and folate for 2 years [41]. However, another study reported that reduction of Hcy levels via B_{12} supplementation in healthy elderly appeared to have no effect on markers of bone turnover in a 2-year study [42]. Two-month supplementation with folate in healthy individuals led to a decrease in Hcy, but no effect on bone turnover markers was found [43]. Thus, the results of these clinical studies and observations are still inconclusive with respect to the effectiveness of B_{12} supplementation in maintaining bone health.

It will be of most relevance to examine whether reducing Hcy and MMA in a normal aging population may prevent the occurrence of osteoporosis. Further research and intervention studies will be required to determine to what extent B_{12} and folate supplementation may prevent bone loss by reducing Hcy- and MMA-induced osteoclast activity during aging and in case of B_{12} deficiency.

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