Increased Osteoclast Activity in the Presence of Increased Homocysteine Concentrations

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Background: Increased plasma homocysteine (HCY) may be an independent risk factor for osteoporotic fractures and therefore may also adversely affect bone metabolism. We analyzed the effect of HCY on human osteoclast (OC) activity.

Methods: We cultured peripheral blood mononuclear cells from 17 healthy male donors [median (SD) age, 30 (5) years] for 20 days with 25 μg/L macrophage-colony-stimulating factor (days 0–11), 20 μg/L receptor-activator of nuclear factor-κB ligand (days 6–20), and 4 different concentrations of HCY (0, 10, 50, and 100 μmol/L; days 0–20). For control purposes, cysteine and glutathione were tested in equimolar concentrations. OCs were identified as large, multinucleated cells with tartrate-resistant acid phosphatase (TRAP) activity and surface vitronectin receptors. We quantified OC activity by measuring TRAP activity. We analyzed cathepsin K (CP-K) activity in 9 donor samples and estimated the dentine-resorbing activity on standard dentine slices in 3 samples.

Results: After 20 days of culture, most cells were fully differentiated OCs. TRAP activity increased with increasing HCY concentrations (P < 0.001). HCY concentrations of 10, 50, and 100 μmol/L stimulated TRAP activity by 20%, 15%, and 42%. Additionally, HCY stimulated CP-K activity (P = 0.005): in the presence of 100 μmol/L HCY, CP-K activity was ~38% higher than in controls (P = 0.002). Bone-resorbing activity was significantly increased in cultures with 50 and 100 μmol/L HCY. Cysteine and glutathione significantly decreased TRAP and CP-K activity.

Conclusions: Increased HCY concentrations specifically stimulate OC activity in vitro, suggesting a mechanistic role of HCY for bone resorption. Future studies clarifying the mechanistic role of increased HCY concentrations in osteoporosis could have interesting therapeutic implications.

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Osteoporosis is a major public health problem, causing considerable morbidity and mortality (1–3). It has been estimated that in a 10-year period, postmenopausal white women in the United States will experience 5.2 million fractures of the hip, spine, or distal forearm, which will lead to 2 million person-years of fracture-related disability (4). Annual expenditures for the direct medical care of osteoporotic fractures in the United States in 1995 totaled $13.8 billion, or $17.5 billion adjusted to 2002 (5). A comparable situation can be found in Europe and other industrialized countries (6, 7). Prevention of osteoporosis by identifying risk factors, or risk indicators, is therefore an important issue. Previous studies identified advancing age, female sex, early menopause, low body weight, cigarette smoking, alcohol consumption, low calcium intake, low physical activity, tallness, previous low-trauma fracture as an adult, and history of hip fracture in a first-degree relative as important risk factors for osteoporosis (8–10).

Recently, increased plasma homocysteine (HCY)4 has been suggested to be an independent risk factor for osteoporotic fractures in elderly persons (11, 12). Little is known, however, about the mechanistic role of HCY in osteoporosis. A link between HCY and bone disease was

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Received April 27, 2005; accepted September 6, 2005.
Previously published online at DOI: 10.1373/clinchem.2005.053363

*Nonstandard abbreviations: HCY, homocysteine; OC, osteoclast; BMD, bone mineral density; PBMC, peripheral blood mononuclear cell; TRAP, tartrate-resistant acid phosphatase; CP-K, cathepsin K; PBS, phosphate-buffered saline; α-MEM, α-minimum essential medium; RANKL, receptor-activator of nuclear factor-κB ligand; and VNR, vitronectin receptor.
first made in 1966, when McKusick (13) hypothesized a disturbed collagen cross-linking in patients with homocystinuria. Two later studies supported this hypothesis (14, 15). However, it is unclear whether these results from patients with the congenital condition of homocystinuria, characterized by extremely high plasma HCY concentrations, are directly applicable to normal variations of HCY among adults.

Moderate hyperhomocysteinemia among adults is caused mainly by folate or vitamin B12 deficiency (16, 17). Several studies found that cobalamin and folate status are related to bone mineral density (BMD) and fracture risk (18–28). In a 2-year prospective, placebo-controlled, double-blind trial, Sato et al. (29) observed a strong reduction in fracture incidence in stroke patients who received high doses of folate and vitamin B12. In addition, low cobalamin status has been shown to reduce osteoblast activity (30). Nothing is known, however, about osteoclast (OC) function in the presence of increased HCY or low B-vitamin concentrations. We hypothesized that increased HCY concentrations stimulate OC activity, leading to increased collagen I breakdown. Accordingly, we analyzed the influence of HCY on human OC activity in vitro.

**Materials and Methods**

**STUDY DESIGN**

We collected venous blood from 17 healthy male donors with a mean (SD) HCY concentration of 10.4 (1.6) μmol/L into EDTA-containing tubes (Sarstedt). Blood donors had a median (SD) age of 30 (5) years, a mean (SD) weight of 76 (9) kg, and mean height of 179 (6) cm. None of the participants used pharmaceuticals or vitamin supplements.

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA blood and cultured to fully differentiated OCs in the presence of various HCY concentrations. To demonstrate the specificity of an HCY-mediated effect, we tested as controls the related thiols cysteine and glutathione. For control cultures with cysteine and glutathione, we used RANKL (Sigma), osteocalcin (Sigma), and vitronectin receptor-positive (VNR+) multinucleated cells (containing ≥3 nuclei). TRAP staining was done as described previously by Benito et al. (31), with pure, cold acetone and 990 mL/L ethanol (1:1 by volume) as fixative. For VNR detection, 23C6 monoclonal antibody (Serotec) diluted 1:40 in PBS was the primary antibody and a fluorescein isothiocyanate-marked sheep anti-mouse IgG antibody (Serotec) diluted 1:60 in PBS was the detection antibody.

**QUANTIFICATION OF OC ACTIVITY**

OC activity was quantified by TRAP, CP-K, and dentine-resorbing activity, which detect 3 independent domains of OC function.

We first measured TRAP activity with the ACP method (Roche Diagnostics), adapted to a 96-multiwell plate. Cells were fixed for 1 min with a 1:1 mixture (by volume) of cold, pure acetone (Sigma) and 990 mL/L ethanol (Sigma). We then added 100 μL of an acidic 1-naphthylphosphate substrate/fast red TR® chromogen solution, enriched with 100 mmol/L sodium tartrate (provided as a tablet in the reagent set), to each well. The 1-naphthylphosphate substrate/fast red TR chromogen solution consisted of 150 mmol/L citrate buffer (pH 4.8), 12.1 mmol/L 1-naphthylphosphate, 1.2 mmol/L fast red TR salt, and 220 mmol/L 1,5-pentanediol. After the addition of this solution to the fixed cells, 1-naphthylphosphate was cleaved by the TRAP enzyme into 1-naphthol and phosphate. The fast red TR and 1-naphthol then formed a red azo dye that could be detected photometrically. After a 10-min incubation, we measured the absorbance at 405 nm, using a Polarstar photometer (BMG Labtech). In several control experiments with and without tartrate addition, we confirmed that the residual acid phosphatase activity measured with our TRAP assay was actually tartrate resistant.

Because collagen resorption is mediated mainly by...
CP-K, we quantified the CP-K activity of cultured OCs by a commercial assay (Biovision). The principle of this assay is the cleavage of an amino-4-trifluoromethyl coumarin-labeled Z-leucine-arginine substrate, the preferred substrate of CP-K. The released amino-4-trifluoromethyl coumarin is then quantified by a fluorometer equipped with a 400 nm excitation filter and a 505 nm emission filter.

TRAP and CP-K activity are indirect indicators of OC function. We therefore completed our data by assessing the dentine-resorbing activity of cultured OCs on standard dentine discs with a thickness of 0.3 mm (IDS). Thirty-six dentine discs were placed in the wells of a 96-multiwell plate. PBMCs from 3 donors were cultured, as described before, in these wells (3 slices per donor and HCY concentration). After 20 days, the culture medium was removed, and the dentine slices were washed 3 times with 200 μL of PBS (PAA Laboratories GmbH). Adherent cells were removed by immersing the dentine slices for 10 s in 1 mol/L sodium hypochlorite solution. Finally, we stained the dentine slices for 3 s in a 10 g/L toluidine blue solution (Sigma Aldrich). After we dried the slices with a paper towel, we used light microscopy to analyze the bone-resorbing activity.

STATISTICAL ANALYSIS
The descriptive statistics provide data as the mean (SD). Means were compared with a Student t-test (comparison of 2 means) or a one-way ANOVA (more than 2 means; e.g., TRAP activity at 0, 10, 50, and 100 μmol/L HCY) with a least-significant-difference post hoc test. Results of TRAP and CP-K activity measurements from each donor are expressed as a percentage of the corresponding control activity (mean of 8 control wells without addition of HCY, cysteine, or glutathione). A P value <0.05 was considered significant. Calculations were done with the software package SPSS (Ver. 11.0 for windows; SPSS Inc).

Results
After 20 days of culture, most of the cells were fully differentiated OCs (Fig. 1). Cells were characterized as fully differentiated OCs only if they fulfilled all 3 of the following criteria: (a) large multinucleated cells with ≥3 nuclei; (b) TRAP+ (red color); (c) VNR+ (circular fluorescence pattern).

TRAP ACTIVITY
Absolute TRAP activity exhibited large interindividual variation, which was mainly attributable to differences in growth behavior. The mean (SD) absorbance (A) of control cultures (without HCY) from all 17 donors was 0.501 (0.350; range, 0.253–1.78). We therefore expressed TRAP activity as a percentage of controls, using the mean absorbance of control cultures without HCY (n = 8 per individual) as 100%.

TRAP activity increased with increasing HCY concentrations (P <0.001; Fig. 2). HCY concentrations of 10 and 50 μmol/L induced 15%–20% higher TRAP activity than controls (Fig. 2). At 100 μmol/L HCY, TRAP activity further increased by 42% (P <0.001). In addition, there were significant differences in TRAP activity between 10 and 100 μmol/L HCY (P <0.001) as well as between 50 and 100 μmol/L HCY (P <0.001). We observed no difference between 10 and 50 μmol/L HCY (P = 0.325). The HCY-related thiols cysteine and glutathione did not induce an increase in TRAP activity. Contrary to the effect of HCY, both cysteine and glutathione caused significant decreases in TRAP activity (Fig. 2).

CP-K ACTIVITY
CP-K activity exhibited large interindividual variations comparable to those of TRAP. The mean (SD) CP-K activity in the 9 donors investigated was 28 525 (17 157) relative fluorescence units (range, 5970–46 017). We

![Fig. 1. Fluorescence and light photomicrographs of VNR+ (A) and TRAP+ OCs (B) cultured in macrophage-colony-stimulating factor– and RANKL-supplemented α-MEM after 20 days of culture.](image)

![Fig. 2. Mean (95% confidence interval; error bars) percentage of OC TRAP activity after 20 days of culture in the presence of different concentrations of HCY, cysteine, and glutathione. All values are relative to the mean for controls (no HCY added to the culture medium). P <0.001 for comparison of all groups (one-way ANOVA), # P <0.05 (LSD post hoc test); **, P = 0.01 (LSD post hoc test).](image)
therefore expressed CP-K activity as a percentage of control culture activity, setting the mean relative fluorescence units of control cultures without HCY (n = 8 per individual) as 100%.

CP-K activity increased significantly with higher HCY concentrations (P < 0.003; Fig. 3). In the presence of 100 μmol/L HCY, CP-K activity was ~38% higher than in controls (P = 0.002). There were also significant differences in CP-K activity between 10 and 100 μmol/L HCY (P = 0.007) as well as between 50 and 100 μmol/L HCY (P = 0.003). We observed no changes between controls and HCY at concentrations of 10 (P = 0.671) and 50 μmol/L (P = 0.860). In contrast to the effect of HCY, cysteine and glutathione induced significant decreases in CP-K activity (Fig. 3).

**Dentine-resorbing activity**

The surface of untreated dentine discs is very homogeneous. In the presence of active OCs, this homogeneous surface became eroded, and resorption pits were formed. The dentine-resorbing activities of the cultured OCs under control conditions and with 10 μmol/L HCY were comparable (Fig. 4). Higher concentrations of HCY caused a considerable increase in the number and sizes of the resorption pits. The higher number and the increased diameter of the resorption pits indicated increased dentine-resorbing activity.

**Discussion**

The main finding of this study was that human OC activity increased significantly in the presence of increased HCY concentrations but not in the presence of equimolar concentrations of the related thiols cysteine and glutathione. HCY stimulated 3 different functional domains of the cultured OCs: TRAP, CP-K, and dentine-resorbing activity. Our results suggest a relevant mechanistic role of HCY in bone metabolism, mediated by OCs.

TRAP contributes to the intracellular processing of primary bone matrix degradation products and is finally released through the basolateral membrane of resorbing OCs (32–34). The enzyme has been used as a marker of OC function for more than 20 years (35) and has been shown to be a specific and sensitive indicator of bone resorption (36–41). In the present study, HCY dose-dependently increased TRAP activity over a broad clinically relevant range, indicating a stimulatory effect of HCY on OC activity. However, TRAP is only indirectly involved in bone resorption (32–34). Bone resorption by OCs is caused mainly by acid (inorganic matrix) and lysosomal proteases (organic matrix), which are released through the ruffled border area into the space between the cell membrane and extracellular bone matrix (resorption lacuna). The most important protease involved in this process is CP-K (32–34). Our data indicate that in the presence of 100 μmol/L HCY, CP-K activity in cultured OCs increased significantly, by ~40%. This finding supports the results observed for TRAP measurement. Lower HCY concentrations of 10 and 50 μmol/L did not modify CP-K activity, possibly because of interindividual variations in CP-K activity and the relatively low number of study participants. Interindividual variations in TRAP and CP-K are caused mainly by constitutional differences in cell growth and OC formation. To confirm the quanti-
tative results obtained for TRAP and CP-K, we cultured OCs on standard dentine discs. HCY at 50 and 100 μmol/L increased the number and the sizes of resorption pits (Fig. 4), indicating increased activity of the cultured OCs. Because resorption pits at higher HCY concentrations exhibited a strong tendency to fuse, quantitative analysis by counting the resorption pits was not meaningful. The present study shows concordant results with 3 independent markers of OC activity, indicating a stimulatory effect of clinically relevant HCY concentrations on OC activity. The specificity of the stimulatory effect of HCY on OC activity is shown by the significant decreases in TRAP and CP-K activity in cultures with equimolar concentrations of the related thiol cysteine and glutathione. Even if we do not have a mechanistic explanation for the inhibitory effect of cysteine and glutathione, these results exclude a general group effect of thiols on OC activity.

A mechanistic role of HCY in bone metabolism is known to occur in patients with homocystinuria, a genetic disorder characterized by severe hyperhomocysteinemia (13–15, 42). It has been suggested that disturbed cross-linking of collagen fibrils and reduced fibrillin-1 and -2 deposition lead to a disturbed architecture of bone matrix in homocystinuric patients (43, 44). The impact of intermolecular collagen cross-links in bone has been adduced, in part, from studies of lathyrism (45). The sweet pea (Lathyrus odoratus) compound β-aminopropionitrile irreversibly inhibits lysyl oxidase and blocks initial collagen cross-link formation. This effect could lead to reduced bone quality and might be an alternative explanation for an increased fracture risk in hyperhomocysteinemic patients. The only existing study in humans, however, analyzed several biochemical markers of collagen formation and breakdown in 10 pediatric patients with homocystinuria (14). In that study, homocystinuric patients had lower serum β-collagen cross-links (cross-linked C-terminal telopeptides of collagen I) than healthy controls. No differences were seen for bone formation markers. Because of the relatively high interindividual variation of most biochemical bone markers and the low number of participants, the impact of these results is strongly limited. In addition, the conclusion that lower concentrations of cross-linked C-terminal telopeptides of collagen I in homocystinuric patients indicate a disturbed collagen cross-linking is highly speculative and is not substantiated by further evidence. Because the opposite has not been demonstrated, however, McKusick’s hypothesis (13) has to be considered as a possible mechanism that needs further exploration. Previous animal studies have been performed mainly in fast-growing chickens (15, 43). These studies found a stimulation of bone growth, a lower BMD, and an altered bone matrix composition in the presence of increased HCY concentrations. Mechanical bone strength was not changed, however. In contrast, 2 recent studies with more than 4000 patients reported an increased risk for osteoporotic fractures in elderly persons with moderate hyperhomocysteinemia (11, 12). Moreover, in adults, BMD has no, or only a weak, association with HCY (12, 25). It is therefore reasonable to speculate that findings from individuals with homocystinuria or animals with a growing skeleton cannot directly be transferred to normal variations of HCY in elderly individuals. Currently, there are no published reports of analysis of the mechanistic role of normal variations of HCY in individuals without homocystinuria.

Folate and vitamin B12 deficiencies are the most common causes of increased HCY concentrations in elderly individuals. Several studies found that cobalamin and folate status are related to BMD and fracture risk (18–28). In addition, low cobalamin status has been shown to decrease osteoblast activity (30). In a recent prospective intervention trial, fracture rates were much lower in stroke patients treated with high doses of folate and vitamin B12 (29). There are no studies, however, analyzing the role of HCY, folate, and vitamin B12 on OC activity. It is not clear whether folate, vitamin B12, and HCY affect bone metabolism by the same mechanism. If they do, then the measurement of HCY might be sufficient to screen folate and vitamin B12 status. If these vitamin act by different pathways, however, then the detection of all components is important to assess individual risk for adverse effects on bone metabolism.

In conclusion, the present study demonstrates that increased HCY concentrations stimulate OC activity in vitro, suggesting a mechanistic role of HCY for bone resorption. Future studies clarifying the mechanistic role of increased HCY concentrations in osteoporosis could have interesting therapeutic implications because HCY-lowering therapy could be beneficial for osteoporotic patients.

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