

# Homocysteine attenuates the expression of osteocalcin but enhances osteopontin in MC3T3-E1 preosteoblastic cells

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## Abstract

It has been pointed out that very high plasma levels of homocysteine are characteristic of homocystinuria, a rare autosomal recessive disease accompanied by the early onset of generalized osteoporosis. However, it is unclear by which mechanism hyperhomocysteine induces osteoporosis, although it is known to interfere with the formation of cross-links in collagen, an essential process in bone formation. Therefore, we investigated the effect of homocysteine on expression of osteocalcin and osteopontin in MC3T3-E1 preosteoblastic cells. Confluent cells were grown in RPMI 1640 containing 10% fetal calf serum with or without homocysteine in an atmosphere of 95% humidified air, 5% CO<sub>2</sub> at 37 °C. The secretion of osteocalcin from the cells increased time-dependently until the end of culture (day 34), but 500 μM homocysteine led to an approximately 61% decrease for osteocalcin after 19 days of culture as compared with the control. On the other hand, osteopontin was not inhibited by 500 μM homocysteine but rather activated, and ranged from 134%–209% of the control level in the period from 10 days until the end of culture. From analysis of RT-PCR for mRNA of osteocalcin and osteopontin at the end of the culture, homocysteine levels of 100 and 500 μM significantly increased the expression of osteopontin mRNA with the control ( $p < 0.05$ ). In contrast, the expression of osteopontin mRNA was suppressed in a dose-dependent manner, showing a mirror image of the effect on osteopontin mRNA. These findings suggest that hyperhomocysteinemia appears to be an independent risk factor for osteoporosis by disturbing osteoblast function.

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## 1. Introduction

Osteoporosis is a systemic bone disorder characterized by low bone mass and microarchitecture in the bone tissue, which leads to reduced strength and increased risk of fracture. In general, it is well known that osteoporosis is associated with many etiological causes such as nutrition, cytokines, hormones, and aging. Recently, Meurs et al. reported that the association between homocysteine levels

and the risk of fracture appeared to be independent of bone mineral density and other potential risk factors for fracture [1]. In fact, it has been pointed out previously that very high plasma levels are characteristic of homocystinuria, a rare autosomal recessive disease accompanied by the early onset of generalized osteoporosis [2,3]. However, it is unclear by which mechanism hyperhomocysteine induces osteoporosis, although it is known to interfere with the formation of cross-links in collagen [4], an essential process in bone formation. On the other hand, osteocalcin and osteopontin, non-collagenous extracellular proteins, are regarded as markers of osteoblasts and play the important roles in bone formation [5,6]. In the present study, we therefore inves-

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tigated the effects of homocysteine on expression of osteocalcin and osteopontin in a well-characterized osteoblast precursor cell line, MC3T3-E1, derived from newborn mouse calvaria [7]. The results presented here demonstrate for the first time that homocysteine reduces the osteocalcin mRNA level and osteocalcin protein but increases osteopontin in MC3T3-E1 cells.

## 2. Materials and methods

### 2.1. Cell culture

MC3T3-E1 cells (a clonal preosteoblastic cell line derived from newborn mouse calvaria) were grown in RPMI 1640 (Sigma, St. Louis, USA) containing 10% fetal calf serum (Irvine Scientific, Santa Ana, California, USA) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin; BioWhittaker, Walkersville, Maryland, USA) in an atmosphere of 95% humidified air, 5% CO<sub>2</sub> at 37 °C. After the cells had become confluent (approximately 2 × 10<sup>6</sup> cells/10 cm-diameter culture dish), the medium was replaced with the RPMI 1640 medium supplemented with 100 and 500 µM homocysteine (Wako Pure Chemicals Co., Osaka, Japan) for 34 days. The medium was changed daily for 3 days and then every 3 days. Conditioned media from the indicated time periods of the culture were stored at –30 °C until assay.

### 2.2. Cell viability

At the end of culture (34 days) cell viability was determined by using a microtiter assay for the formation of formazan from Cell Counting Kit-8 (Dojindo Chem., Kumamoto, Japan) as described previously [8].

### 2.3. Analysis of cell morphology

At each change of the medium, the cells in the culture plate were observed by phase-contrast microscopy using an FC-300Z/OL (Olympus, Tokyo, Japan).

### 2.4. Measurement of osteocalcin and osteopontin

The contents of osteocalcin and osteopontin in cultured medium of MC3T3-E1 cells were measured using ELISA Kits of Biomedical Technologies Inc. (MA, USA) and Immuno-Biological Lab. Co. (Gunma, Japan), respectively.

### 2.5. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using ISOGEN (Nippon Gene, Toyama, Japan) from MC3T3-E1, as instructed by the manufacturer. First-strand cDNA was synthesized from the total RNA using ReverTra Ace Moloney murine leukemia virus reverse transcriptase (TOYOBO, Osaka, Japan) and

random primers (TOYOBO), and was subjected to PCR amplification with AmpliTaq Gold DNA Polymerase (Roche Molecular Systems, Inc., Branchburg, New Jersey, USA) using specific PCR primers: mouse osteocalcin, 5'-ATG AGG ACC CTC TCT CTG CT-3' and 5'-CCG TAG ATG CGT TTG TAG GC-3'; mouse osteopontin, 5'-CTT GCT TGG GTT TGC AGT CT-3' and 5'-AAA TGC AGT GGC CGT TTG CA-3'; mouse glyceraldehyde-3-phosphate dehydrogenase (G3PDH), 5'-CGG AGT CAA CGG ATT TGG TCG TAT-3' and 5'-AGC CTT CTC CAT GGT GGT GAA GAC-3'. The PCR products were separated by electrophoresis on 1% agarose gel. All PCRs were performed in triplicate. Quantitation was achieved using the comparative threshold cycle method according to the manufacturer's protocol.

### 2.6. Statistic analysis

For statistical calculation the Student's *t*-test was used. Data are given as mean and standard deviation (mean ± S.D.). A significant difference was recognized when *p* < 0.05.

## 3. Results

### 3.1. Effects of homocysteine on secretion of osteocalcin and osteopontin in MC3T3-E1 cells

Osteocalcin is a noncollagenous bone-specific matrix protein. As shown in Fig. 1, osteocalcin content of the medium of MC3T3-E1 cells in the absence of homocysteine increased time dependently until the end of culture (day 34). In cells treated with homocysteine, 500 µM homocysteine

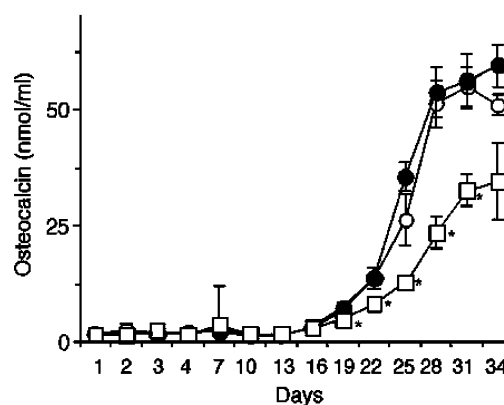


Fig. 1. Time course of the effect of homocysteine on osteocalcin secretion in MC3T3-E1 cells. Confluent cells (approximately 2 × 10<sup>6</sup> cells/10 cm-diameter culture dish) were cultured with RPMI 1640 medium supplemented with 100 µM and/or 500 µM homocysteine for the time periods indicated. The amounts of osteocalcin in MC3T3-E1 cell culture medium were measured by ELISA as described in the text. Data are shown as the mean ± SD from triplicate cultures. \*Data show a significant difference when compared to the control at each time point (*p* < 0.05). Control (open circles); 100 µM homocysteine (filled circles); 500 µM homocysteine (squares).

led to an approximately 61% decrease of osteocalcin after 19 days of culture as compared with the control, in spite of showing no effect until 16 days of culture. The inhibition of osteocalcin secretion by the homocysteine, approximately from 46% to 64%, continued until the end of culture (day 34). In contrast, osteopontin was not inhibited by 500  $\mu\text{M}$  homocysteine but rather activated into the range from 209% to 134% after 10 days and until the end of culture (Fig. 2). However, 100  $\mu\text{M}$  homocysteine did not affect secretion of osteocalcin during the culture period, whereas osteopontin was significantly increased on day 34.

### 3.2. Effects of homocysteine on cell morphology

To clarify the mechanism for secretion of osteocalcin and osteopontin in MC3T3-E1 cells treated with homocysteine, we investigated morphological changes of cells. As shown in Fig. 3, more healthy MC3T3-E1 cells with intact morphology were observed up to the concentration of 500  $\mu\text{M}$  homocysteine. Similarly, cell viability on the end of culture (day 34) showed no significant differences compared with the control cells ( $103.9 \pm 1.7\%$  for 100  $\mu\text{M}$  homocysteine;  $104.8 \pm 2.0\%$  of the control for 500  $\mu\text{M}$  homocysteine). However, mineralized nodule formation was not observed for the time periods in the presence and absence of homocysteine, because the medium did not contain  $\beta$ -glycerophosphate or ascorbic acid.

### 3.3. Effects of homocysteine on the mRNA expression of osteopontin and osteocalcin in MC3T3-E1 cells

As demonstrated in Figs. 1 and 2, MC3T3-E1 cells grown in the presence of homocysteine showed different changes in

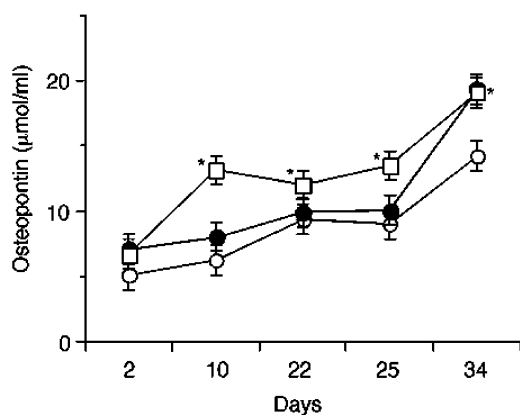


Fig. 2. Time course of the effect of homocysteine on osteopontin secretion in MC3T3-E1 cells. Confluent cells (approximately  $2 \times 10^6$  cells/10 cm-diameter culture dish) were cultured with RPMI 1640 medium supplemented with 100  $\mu\text{M}$  and/or 500  $\mu\text{M}$  homocysteine for the time periods indicated. The amounts of osteopontin in MC3T3-E1 cell culture medium were measured by ELISA as described in the text. Data are shown as the mean  $\pm$  SD from triplicate cultures. \*Data show a significant difference when compared to the control at each time point ( $p < 0.05$ ). Control (open circles); 100  $\mu\text{M}$  homocysteine (filled circles); 500  $\mu\text{M}$  homocysteine (squares).

osteoblast markers such as osteocalcin and osteopontin during maturation. Therefore, we next examined the effects of homocysteine on the mRNA expression of osteopontin and osteocalcin by RT-PCR using MC3T3-E1 cells at the end of culture. As shown in Fig. 4, homocysteine at doses of 100 and 500  $\mu\text{M}$  significantly increased the expression of osteopontin mRNA as compared with the control ( $p < 0.05$ ). In contrast, the expression of osteocalcin mRNA was suppressed in a dose-dependent manner, showing a mirror image of the effect on osteopontin mRNA.

## 4. Discussion

Hyperhomocysteinemia has been reported to be associated with skeletal abnormalities and osteoporosis [1–3,9]. In general, osteoporosis is caused by an imbalance of the functions of osteoblasts and osteoclasts. Therefore, a possible explanation could be that high levels of homocysteine can potentially disturb osteoblasts. Strangely, however, we have rarely seen reports concerning the direct action of homocysteine on osteoblasts, although many investigators have examined the actions of homocysteine with regard to various cells such as monocytes, hepatoma cells, and endothelial cells [10–12]. Therefore, we investigated the effect of homocysteine on osteoblast function using MC3T3-E1 cells, a clonal preosteoblastic cell line derived from newborn mouse calvaria [7]. In general, it is known that osteoblasts produce alkaline phosphatase, type I collagen, Ca-deposition, osteocalcin, and osteopontin [13]. For osteoblast differentiation in this study, we analyzed osteocalcin and osteopontin, which are secreted into the medium from the osteoblasts. The cells time-dependently produced osteocalcin and osteopontin after confluence, as a result of activation of specific genes associated with the osteoblastic phenotype [6]. Thus, this study design was adequate for investigating the effect of homocysteine on osteoblast function. In the present study, we demonstrated for the first time that homocysteine inhibited production of osteocalcin but increased osteopontin. In addition, we clarified homocysteine decreased the osteocalcin mRNA level but increased osteopontin mRNA without affecting cell viability. However, RPMI 1640 medium used in this experiment led to the failure of mineralization in MC3T3-E1 cells, because it did not have ascorbic acid which was required in the mineralization process [7]. Previously, many investigators have shown that homocysteine affects cellular functions, such as induction of the expression and synthesis of physiological substances, based on either directly or via generation of reactive oxygen species [10,14,15]. Therefore, it is possible that ascorbic acid contained in MEM medium interferes with the action of homocysteine, because ascorbic acid plays an important role as antioxidant against reactive oxygen species [16]. Besides, reduced glutathione, which is contained in RPMI 1640 medium but not in MEM, seem to be involved in cellular uptake of homocysteine [17]. In fact,

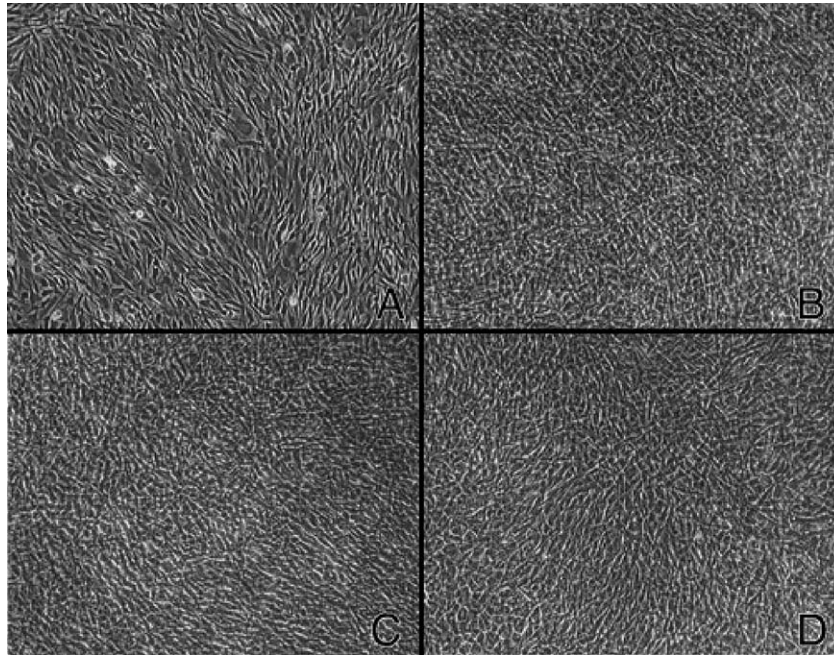


Fig. 3. Phase-contrast micrographs of MC3T3-E1 cells treated with homocysteine. Confluent MC3T3-E1 cells ( $2 \times 10^6$  cells/dish) treated with homocysteine for 34 days were photographed with a phase-contrast light microscope. A, untreated MC3T3-E1 cells at starting point; B, MC3T3-E1 cells after 34 days of culture in the absence of homocysteine; C, MC3T3-E1 cells after 34 days of culture in the presence of 100  $\mu\text{M}$  homocysteine; D, MC3T3-E1 cells after 34 days of culture in the presence of 500  $\mu\text{M}$  homocysteine.

many investigators have used RPMI 1640 medium to examine the effect of homocysteine on the culture experiments of various cells [10,11,15]. Thus, we tried to use RPMI 1640 medium instead of MEM [13].

Regarding the homocysteine concentrations, plasma level of hyperhomocysteine may reach values of 16–30  $\mu\text{M}$  (moderate), 31–100  $\mu\text{M}$  (medium) or greater than 100  $\mu\text{M}$  (severe), values as high as 500  $\mu\text{M}$  found in patients with homocystinuria [14]. In addition, very high plasma homocysteine levels are characteristic of homocystinuria, a rare autosomal recessive disease accompanied by the early onset of generalized osteoporosis [3]. However, the pathophysiological mechanism for the occurrence of early osteoporosis in homocystinuria patients is not completely

understood. Therefore, we investigated the effect of homocysteine on the function of osteoblasts using homocysteine at doses of 100 and 500  $\mu\text{M}$ , which were used to clarify for augmentation of superoxide anion release and NADPH oxidase in human neutrophils [15] and induction of MCP-1 and IL-8 secretion in human monocytes [10]. Consequently, we could demonstrate for the first time that higher concentration of homocysteine in homocystinuria patients may lead to disturbance of osteoblast function, resulting in increase of the expression for osteopontin mRNA in contrast to osteocalcin mRNA.

Regarding the roles of osteopontin and osteocalcin in osteoblast function, they are not very clear. Previously, Audi et al. reported that osteocalcin is a late marker of

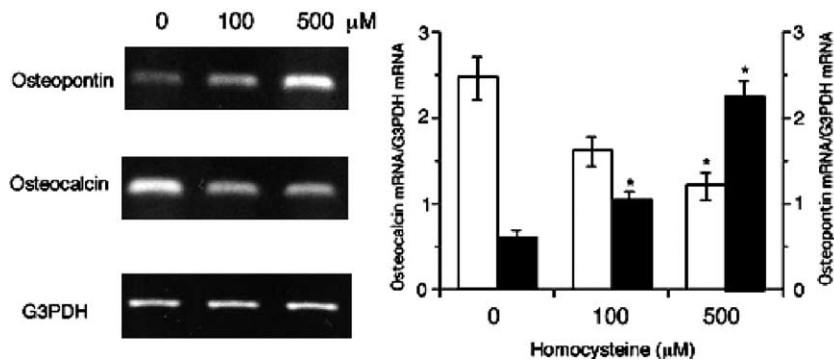


Fig. 4. Effects of homocysteine on the mRNA expression of osteopontin and osteocalcin in MC3T3-E1 cells. MC3T3-E1 cells were exposed to 100 and/or 500  $\mu\text{M}$  homocysteine for 34 days after confluence. Relative levels of osteopontin and osteocalcin mRNA were measured by real-time PCR and standardized by glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA. The values were normalized to G3PDH mRNA level of each sample. Each bar represents the mean  $\pm$  SD from triplicate cultures. White bar: osteocalcin mRNA; black bar: osteopontin mRNA. \*Data show a significant difference when compared to the control at each concentration ( $p < 0.05$ ).



osteoblast differentiation that is related closely to osteoblast maturation [6]. On the other hand, osteopontin has been demonstrated to play a role as an inhibitor of enhanced bone formation or crystal growth *in vivo* as well as *in vitro* [18,19]. Moreover, Ishii et al. recently reported that the addition of osteopontin increased the expression of RANKL and augmented differentiation of osteoclasts from osteopontin-deficient cells [20]. However, it remains the effect on osteoblast function for homocysteine levels within the normal or slightly elevated range (16–30  $\mu\text{M}$ ) in either RPMI 1640 medium or MEM supplemented with ascorbic acid and inorganic phosphate. These points should be further investigated together with the effect of homocysteine on bone density *in vivo*. Taken together, it is conceivable that the hyperhomocystenemia appears to be an independent risk factor for osteoporosis by disturbing of osteoblast function.

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