# A Study of Correlation of Osteoblasts from Peripheral Blood with Related Bone Turnover Markers

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*The bone remodeling process called osteoblasts has an important role in bone formation working together with osteoclasts of which the cells are responsible for bone resorption. In addition, these bone turnover markers are used to follow-up the conditions of bone remodeling in the patients. Recently, osteoblastic lineage cells have been found that they exist in the human peripheral blood. However, there has been no report about the amount of circulating osteoblastic lineage cells that have the relationship with the samples of bone turnover markers showing the bone remodeling condition. In the present study, circulating osteoblasts were quantified in 43 subjects aged between 25-90 years. They were classified by age into 3 groups: A) lower than 60 years old (n = 9), B) from 60 to 79 years old (n = 22) and C) equal and over 80 years old (n = 12). All were studied by the flow cytometry method using an antibody to osteocalcin and bone turnover markers β-CrossLab (βCTx), PINP and NMID. These markers including parathyroid hormone were analyzed. The result showed the best positive correlation between the percentage of circulating osteoblasts and bone turnover markers of the equal and over 80-year-old group. While another result exhibited the negative correlation of circulating osteocalcin positive cells with the bone turnover markers in the group of lower than 60 years old. As circulating osteoblasts had the correlation with bone turnover markers in the group aged ≥ 80 years old, this could be used as the markers to follow up the bone turnover situation of the patients in this age group. However, this is a pilot study. Further analysis of more amounts of subjects should be done for a better result.*

***Keywords:*** *Circulating-osteoblast, Circulating-osteocalcin-positive cell, Bone remodeling, Bone marker, bCTx, Flow cytometry analysis*

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Osteoblasts accepted as the bone formation cell work in the coupling process with osteoclasts in bone remodeling. The osteoblast originated from mesenchymal stem cell in bone marrow and synthesized matrix protein[1,2]. Besides, the previous study of osteoblast[3] described that there were existing circulating osteoblast-lineage cells in human peripheral blood in adolescent males and adult males. Its finding showed the correlation with the markers of bone formation that was markedly higher during pubertal growth. They studied the osteocalcin positive and bone specific alkaline phosphatase (AP) positive cells in peripheral blood. In the present study[3], the value of the adolescent males showed that their positive cells for osteocalcin in the circulation had five times increase more than the adult group due to the pubertal growth. This result was correlated with the markers of bone formation that were clearly raised up[3]. Another study[4] showed that circulating osteocalcin positive cells about 46% also expressed alkaline phosphatase and 37% of them were positive for hematopoietic/endothelial marker CD34. The male subjects in the present study[4] exhibited the increasing concentration of circulating osteocalcin positive cells as a function of age while CD34 positive cells tended to decrease according to aging. While the circulating osteocalcin positive cells were increased due to age at least in men, it remained unclear that the origin of these cells played a role either in bone formation or in vascular calcification and probably in both[4]. The relationship between the circulating osteogenic cells defined by hematopoietic lineage negative (lin-)/AP+ in the rapid group and in

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the slow bone loss one was studied in order to investigate the relationship between the circulating osteocalcin positive cells in the postmenopausal women in the rapid and in the slow bone loss groups, Lin-/AP+ cells in peripheral blood of both postmenopausal female groups showed different levels of some mRNAs. They studied both in bone marrow and in peripheral blood. The lin-/AP+ cells in bone marrow were outnumbered the ones in peripheral blood. However, both sources were able to mineralize *in vitro* and be expressed the similar levels of genes, *i.e.* the key roles of osteoblast markers. As a result, the lin-/AP+ cells in peripheral blood were compatible with osteoblastic lineage[5]. The present study in thalassemic patients[6] (aged 26.6 $\pm$ 10.3) found that the circulating osteocalcin positive cells were significantly increased compared to the control group (aged 28.3 $\pm$ 4.1). The percentages of osteocalcin positive cells by the flow cytometric analysis were 3.21 $\pm$ 3.19 and 1.09 $\pm$ 0.53, respectively.

While the process of bone remodeling ostoblasts and ostoclasts worked together and after the bone resorption, the reversal and formation processes of bone remodeling were accompanied by osteoblasts to form bone[7]. Osteoblasts differentiated from mesenchymal as mentioned above resided in both bone marrow and peripheral blood. The existing circulating osteoblasts were studied as described before, and analyzed by the flow cytometry technique. The present study aimed to investigate the circulating osteocalcin positive cells and other bone markers to define the correlation between these cells and the set of bone markers.

## Material and Method

The bone marker NMID, Betacrosslaps (βCTx), Procollagen type I N propeptide *(*PINP*)*, parathyroid hormone (PTH) and circulated-ostocalcin positive cells in volunteers (n = 43) aged between 25-90 years were monitored.

Ten milliliters of peripheral blood were collected for both analyses of bone markers by using $K_2$EDTA as anticoagulant and of circulating osteocalcin-positive cells by using flow cytometry. Peripheral blood mononuclear cells (MNCs) were isolated by Ficoll gradient centrifugation method. The peripheral blood were layered over Isoprep (1.077 g/ml) (Robbins Scientific, Sunnyvale, CA)[6,8] and MNCs were collected and processed for immunostaining by the modified method as described by Eghbali-Fatourechi GZ ( 2007) and Eamwijit T[4,6,8]. Briefly, MNCs collected from interface between the blood and Isoprep were washed three times with phosphate buffer saline (PBS) pH 7.4. MNCs were counted, and then 5 x $10^5$ cells were resuspended in each 2 separated tube fixation buffer (eBioscience, USA) and incubated at room temperature for 10 minutes, later they were washed twice in PBS pH 7.4. The cell pellets were resuspended in permeabilization buffer (eBioscience, USA). After decanting the supernatant, one tube of MNCs was incubated with 10 µl of anti-human osteocalcin-phycoerythrin monoclonal antibody (R & D System Inc., USA) whereas the negative control was incubated with phycoerythrin-labeled mouse IgG1 (R & D System Inc., USA) for 30 minutes at RT in the dark. Then the cells were washed twice with permeabilization buffer and were resuspended in PBS pH 7.4.

The cells in suspension were analyzed by using a Becton Dickinson FACScan cytometer. In the flow analysis, the gate was determined on a forward versus side scatter to set a region around the lymphocyte/monocyte-enriched area. The frequency of positive cells was measured as the percentile of gated cells in fluorescent channels with activities above 99.5% of the corresponding isotype controls, including backgrounds below 0.5%[4-6] as shown in Fig. 1.

## Results

The present study of bone resorption marker in high bone turnover of all age groups (n = 43) exhibited the mean of βCTx was 0.292 ng/ml, SD was 0.196 while the bone formation markers had the mean of NMID was 16.45, SD was 6.464 and the mean of PINP was 30.42, SD was 16.76. The PTH hormone played an important role in calcium homeostasis through their actions on osteoblasts[9]. The mean was 42.14, SD was 22.19. The mean of circulating osteocalcin positive cells was 0.28% and SD was 0.21. The correlations of bone markers, PTH and age with osteoblasts of the group of 25-90 years old between osteocalcin positive cells and bone markers were not highly related as shown in

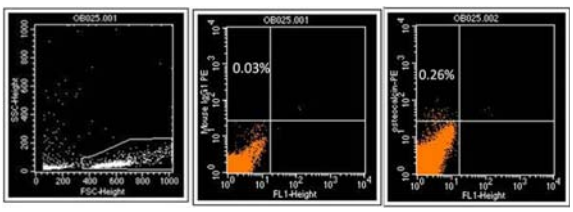


**Fig. 1** Examples of the flow cytometry analysis using the isotype control (middle) and osteocalcin antibody (right)

Table 1.

The data were classified by age into 3 groups: the first group had 9 participants under 60 years old, the second group had 22 participants from 60 to 79 years old and the last one had 12 participants 80 years old and more.

In the under 60-year-old group (the mean = 46.85), the mean of osteocalcin positive cells was 0.30%, SD = 0.15, the mean of βCTx = 0.274 ng/ml, SD = 0.218. The mean range of the bone formation markers NMID and PINP were 16.26 and 33.34 and SD was 5.21 and 14.74, respectively (shown in Table 2). The mean value of PTH was 38.00 and SD was 8.02. The correlation between circulating osteoblast and bone marker was best correlated with PTH (r = -0.65) while the relations between bone formation marker PINP and NMID were moderately related (r = -0.48, r = -0.40 respectively). The other markers had low relation (correlation with CTx = -0.29).

In the second group aged from 60 to 79 years (the mean = 70.67), the mean of circulating osteoblast was 0.24%, SD = 0.22. The mean of the bone resorption marker βCTx was 0.331, SD = 0.207 while the mean of bone formation markers NMID and PINP were 17.57 and 31.51, SD were 7.23 and 19.81, respectively. The Mean of PTH was 47.32 and SD was 27.30. The correlation between osteoblast and bone resorption marker, or βCTX was -0.34 while the correlations with bone formation markers NMID and PINP were -0.37 and 0.18, respectively.

In the last group, aged from 80 and over (the mean = 85.18), the mean percentage of osteoblast was 0.38 and SD was 0.22. The mean of βCTx was 0.243, SD was 0.160 while the mean of bone formation markers

**Table 1.** Mean ± SD of percentage of osteoclcin positive cells, bone turnover marker (βCTx, NMID and PINP) and PTH

| Mean ± SD | %Osteocalcin (+) cell | βCTx (ng/ml) | NMID (ng/ml) | PINP (ng/ml) | PTH (pg/ml) |
|---|---|---|---|---|---|
| All | 0.28 ± 0.21 | 0.292 ± 0.196 | 16.45 ± 6.46 | 30.42 ± 16.76 | 42.14 ± 22.19 |
| < 60 years | 0.30 ± 0.15 | 0.274 ± 0.218 | 16.26 ± 5.21 | 33.34 ± 14.74 | 38.00 ± 8.02 |
| 60-79 years | 0.24 ± 0.22 | 0.331 ± 0.207 | 17.57 ± 7.23 | 31.51 ± 19.81 | 47.32 ± 27.30 |
| ≥ 80 years | 0.38 ± 0.22 | 0.243 ± 0.160 | 14.73 ± 5.70 | 26.64 ± 10.99 | 36.79 ± 15.03 |

**Table 2.** Correlation between bone turnover markers (βCTx, NMID, PINP), PTH and age with the percentage of circulating osteocalcin positive cells (OB)

| Correlation (r) | βCTx:OB | NMID:OB | PINP:OB | PTH:OB | AGE:OB |
|---|---|---|---|---|---|
| All | 0.19 | 0.24 | 0.10 | 0.05 | 0.07 |
| < 60 years | -0.29 | -0.40 | -0.48 | -0.65 | -0.33 |
| 60-79 years | -0.34 | -0.37 | 0.18 | 0.30 | -0.17 |
| ≥ 80 years | 0.46 | 0.55 | 0.61 | -0.21 | -0.20 |

**Table 3.** Percentage of circulating osteocalcin positive cells in each group

| Subject groups | % circulating osteocalcin positive cells |
|---|---|
| All age | 0.28 ± 0.21 |
| < 60 years | 0.30 ± 0.15 |
| 60-79 years | 0.24 ± 0.22 |
| ≥ 80 years | 0.38 ± 0.22 |
| Control group* (age = 28 ± 4.1) | 1.09 ± 0.53 |
| Thalassemia patients* (age 26.6 ± 10.3) | 3.21 ± 3.19 |

*Data from Eamwijit T. The number of circulating osteoblasts in the thalassemia patients. Bangkok: Mahidol University; 2008

NMID and PINP were 14.73 and 26.64 and SD were 5.70 and 10.99, respectively. The correlation with the bone resorption marker named βCTx of which the value was 0.46 was moderate. The correlations with the bone formation marker named NMID and PINP were 0.55 and 0.61 respectively. The mean of PTH was 36.79 and SD was 15.03. PTH had low relation with circulating osteocalcin positive cells (r = -0.21)

**Discussion**

Circulating osteocalcin positive cells increase along with aging in healthy adult males (r = 0.54)[4]. The postmenopausal women had an increasing evidence of circulating osteogenic cells defined by lin-/AP+ marker[5]. The correlation of bone formation marker and osteoblast-lineage cells circulate in significant number and marked higher in pubertal growth[3]. The study in thalassemic patients, circulating osteocalcin positive cells were increased compared with the control group[6]. Osteoblasts play a role in bone formation, in bone remodeling process and in bone resorption and formation that occurs as the coupling effects. The bone marker named βCTx or β-CrossLabs is used as the bone resorption marker. In adult Thai women, the mean of βCTx is 0.310 ng/mL and increases in the menopause group and gradually increases along with aging[10]. In the third group (≥ 80 years old), βCTx had the best correlation with circulating osteoblasts compared with the other groups. This can be concluded that circulating osteoblasts show the relation with resorption situation in this group while exhibit low negative correlation with the < 60-year-old group and the 60-79 year-old groups; the values were -0.29 and -0.34 respectively. The correlations of circulating osteoblasts with both bone formation markers NMID and PINP were also best in the ≥ 80 year-old group. These bone formation markers were moderately related to circulating osteocalcin positive cells (r = 0.55 and r = 0.61 respectively).

Thus, both bone resorption and formation markers were moderately positively related to osteoblasts. The present study exhibited the correlation of osteoblastic lineage in peripheral blood and bone turnover markers only in the ≥ 80-year-old group (Table 2). In this group (≥ 80 years old), the mean of βCTx was the lowest. So were the other bone markers (NMID and PINP) and PTH while the percentage of osteoblastic cells was the highest. The circulating osteoblasts were related to the bone remodeling process especially with the bone formation markers, *i.e.* osteocalcin and bone-specific alkaline phosphatase of the thalassemic patients in the present study by Eamwijit et al[6]. In the present study, the bone turnover markers named βCTx, NMID and PINP exhibit the correlation with osteocalcin positive cells especially in the subjects aged ≥ 80 years old as mentioned above. The study of the thalassemia subjects presented that this disease commonly occurred with bone deformities, scoliosis, chronic bone pain, spontaneous fractures, growth failure nerve compression, osteopenia and osteoporosis[11]. The present study by Eamwijit (2008) showed the increasing percentage of circulating osteocalcin positive cells in the thalassemic patients compared to the control group[6] shown in Table 3. The circulating osteocalcin positive cells were increased not only in bone remodeling process in human but also in vascular injury; however, clear evidences of increasing these cells should be more studied[4]. It is doubtful whether circulating osteoblasts originated in bone marrow and osteoblasts in peripheral blood are related. This concept is still controversial; further study is needed. Although the gene expression of two populations of cells, *i.e.* in the peripheral blood and in bone marrow had some differences, both could conduct mineralization after being cultured in the osteoblast differentiation medium[5]. If these cell populations were originated from the same sources, the circulating osteoblasts in the oldest group may represent the active bone marrow and correlated with βCTx, PINP and NMID displaying the bone turnover situation. As a result, circulating osteoblasts show the best correlation to bone turnover markers with the equal and over 80-year-old group and these bone markers can be used for following-up the bone turnover situation of the patients at this age. Nevertheless, this is a pilot study. More subjects should be investigated for further analysis to achieve better results and conclusion.

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**Potential conflicts of interest**

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## *การศึกษาความสัมพันธ์ระหว่างเซลล์ออสติโอบลาสต์ในกระแสเลือดกับโบนเทิร์นโอเวอร์มาร์คเกอร์ที่เกี่ยวข้อง*

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*ในกลไก bone remodeling ซึ่งเซลล์อ่อนสร้างกระดูก มีบทบาทสำคัญในการสร้างกระดูก จะทำงานสัมพันธ์กับเซลล์ที่มีหน้าที่สลายกระดูก นอกจากนี้ยังมี bone turnover marker สามารถใช้เป็นตัวติดตามสภาวะการสร้างและสลายกระดูกในการตรวจติดตามผู้ป่วย เนื่องจากเซลล์อ่อนสร้างกระดูก จะตรวจพบได้ในกระแสเลือดของมนุษย์ อย่างไรก็ตามยังไม่มีการรายงานการตรวจวัดจำนวนของเซลล์อ่อนสร้างกระดูกว่ามีความสัมพันธ์กับ bone turnover marker ที่ตรวจพบในกลุ่มตัวอย่างที่มี bone remodeling โดยการศึกษานี้จะทำการตรวจวัดจำนวนเซลล์อ่อนสร้างกระดูกในกระแสเลือดของกลุ่มตัวอย่างระหว่างอายุ 25-90 ปี (n = 43) และจัดแบ่งตามอายุออกเป็นสามกลุ่ม 1) กลุ่มที่มีอายุน้อยกว่า 60 ปี (n = 9), 2) กลุ่มที่มีอายุตั้งแต่ 60-79 ปี (n = 22) และ 3) กลุ่มที่มีอายุตั้งแต่ 80 ปีขึ้นไป (n = 12) โดยวิธีทาง Flow Cytometry ด้วยการใช้แอนติบอดีต่อ osteocalcin และวัดระดับของ bone turnover marker ในกระแสเลือด ได้แก่ $\beta$-CrossLab ($\beta$CTx), PINP และ NMID รวมถึงฮอร์โมนพาราไทรอยด์ ผลการศึกษาพบว่าความสัมพันธ์ของปริมาณเซลล์อ่อนสร้างกระดูกมีความสัมพันธ์ ดีที่สุดกับค่า bone turnover marker ในเชิงบวก ของกลุ่มตัวอย่างที่มีช่วงอายุ มากกว่าหรือเท่ากับ 80 ปี แต่มี ความสัมพันธ์ในเชิงลบของกลุ่มตัวอย่างที่มีอายุ น้อยกว่า 60 ปี ปริมาณเซลล์อ่อนสร้างกระดูกมีความสัมพันธ์กับ bone turnover marker ในกลุ่มคนที่มีอายุมากกว่าหรือเท่ากับ 80 ปี อาจใช้เป็นตัวติดตามสภาวะ bone turnover ในกลุ่มผู้ป่วยได้ อย่างไรก็ตามการศึกษานี้เป็นการศึกษานำร่อง จึงควรจะมีการศึกษาเพิ่มเติมในกลุ่มตัวอย่างที่ใหญ่ ขึ้นเพื่อผลการศึกษาที่แม่นยำขึ้น*