Osteocalcin Production By Osteoblast Cultured From Elderly and Young Patients

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ABSTRACT

Study design : This is an experimental study of osteocalcin production by in vitro cultured human osteoblast from elderly and young donors.

Objective : To compare human osteoblast metabolism in different age groups, in cell culture by using osteocalcin production as the osteoblastic marker.

Materials & methods : Cancellous bone was harvested from the proximal tibia of an elderly patient who had total knee arthroplasty (group A) and from a young patient who had closed nailing of tibia (group B). Bure media (no cells) was used as a control group. All bone samples were grown in culture using the standard cell culture technique. The media of the cell cultures were examined for osteocalcin levels using sandwich immunoassay kits for intact human osteocalcin on days 7, 14, and 21. Osteocalcin levels from the elderly patient's osteoblast were compared to the younger patient's osteoblast and both were, in turn, compared to the level in the control media.

Results : Significant and appreciable amounts of osteocalcin were secreted into the culture media. The osteocalcin levels of both the elderly and the young patient's osteoblast media appeared to be higher than the control media. No differences of osteocalcin levels between the elderly and the young patient's osteoblast media on days 7, 14, and 21 could be detected.

Conclusion : There was no difference in osteoblastic metabolism from the elderly and the young human when the cells were grown under the same condition in vitro. This study implies that elderly patient osteoblasts can have normal function if they are grown in proper environment. This study provides basis information regarding utilities of bone tissue culture. The study also suggests that osteoblast derived from cell culture can be utilized regardless of donor age.

INTRODUCTION

As the population ages, certain diseases and medical conditions are more common among the older age group and gaining greater prominence in both public and medical eyes. Osteoporosis is one such condition that is a recognized major public health problem. It affects a great number of people and is associated with numerous bone fracture cases annually. The medical and social costs of osteoporosis, inclusive of direct and indirect costs, continue to rise every year[1].

It is well accepted that osteoporosis results from a basic abnormality in bone remodeling. One generalization would be that osteoporosis is the increase of bone resorption and decrease of new bone formation. In the past the investigations were focused on the bone resorption, but the results have not been conclusive. Today many research efforts are aimed at the regulation of new bone formation. However, these efforts are hampered by the lack of basic information on bone metabolism and bone formation. The assessment of bone metabolism and bone formation remains a difficult problem in clinical medicine. Measurements of alkaline phosphatase in blood and hydroxyproline in urine have been used, each having its limitation in specificity[2].

Osteocalcin, also called bone Gla protein (BGP) and y-carboxy glutamate protein, is a specific product of an osteoblast. It was discovered by Hauschka et al.[3,4] in chicken bone and by Price et al.[5] in bovine. Brown et al[6]. proved that osteocalcin could be used as a marker by showing a positive correlation between the serum osteocalcin concentration and osteoid volume or relative osteoid surface. Osteocalcin is a vitamin K-dependent, calcium-binding protein of bone, the most abundant noncollagenous bone protein, and the seventh most aboundant protein in the human body. This extracellular

protein has a molecular weight of 5,700 D and contains three residues of a calcium-binding amino acid, y-carboxyglutamic acid (Gla).It is synthesized by osteoblast as a 10kD precursor. The Gla residues are formed post-translationally by vitamin K and CO2-dependent carboxylase enzyme complex. In the presence of calcium, the Gla residues allow specific conformational changes and promote osteocalcin binding to hydroxyapatite and subsequent accumulation in bone matrix. A small fraction of the synthesized osteocalcin does not accumulate in bone but is secreted directly into the circulation. The catabolic products of osteocalcin are excreted inthe urine.

Many researchers utilize the serum osteocalcin and urine Gla as markers for osteocalcin production and as indicators to the metabolism and bone formation of the osteoblast, but these measurements are interfered with by many exogenous factors such as drug-induced alterations in osteocalcin's affinity for bone, hormonal status, renal function, age, sex, timing of blood collection, and specificity of the assays used. Osteocalcin and Gla are normally measured by indirect radioimmunoassay methods. To eliminate the interference factors, the osteoblast culture was subsequently developed. In fetal rat calvariae organ cultures, more than 75% of osteocalcin was found in the media[7], however, there were few investigations performed using human osteoblast culture and there are few reports on osteocalcin detection in culture media from human osteoblast culture. Our institute has utilized and adapted the human osteoblast culture technology originally proposed by Shelton[8]. Osteocalcin was detected in culture media using the Sandwich Immunoassay technique[2].

The effect of aging on the metabolism of osteoblast has been investigated by many research groups using serum osteocalcin measurement but the data remains controversial. This study is an experimental study to assess the effect of aging on the metabolism of primary human osteoblast culture from fresh bone specimens.

MATERIALS AND METHODS

Cancellous bone, designated group A, was harvested from the proximal tibia of an elderly patient, a 70-year-old woman who had total knee artheoplasty. The group B sample consisted of bone taken from a 30-year-old woman who had closed nailing of the tibia. Both of the medical histories revealed no metabolic diseases or malignancies. The pure media (no cell) was used as a control group. Each sample was allocated into 30 dishes each. All samples were cultured using cell culture techniques of the Birmingham Medical school[8,9]. The morphology, cell count, calcifying capacity, and osteocalcin production were investigated (Fig. 1). The culture media was replenished weekly. The media from 6 dishes was collected randomly on days 7, 14, and 21 and tested for osteocalcin levels.

There are two approaches for measuring the osteocalcin level. Our approach depends on an immunoassay system using antibodies against bovine osteocalcin while the other involves raising antibodies against the synthetic peptides representing the C-terminal portion of human osteocalcin. The sandwich enzyme immunoassay (EIA) system is known for its high sensitivity and specificity for detecting heterogeneous molecules in the serum. We utilized the EIA method to overcome the problems of limited-region specificity usually associated with comventional radioimmunoassay (RIA).

The sandwich enzyme immunoassay for intact human osteocalcin utilized in this study was developed by Honsoda[2]. (Department of Laboratory Medicine, Tokyo Metropolitan General Hospital).

The tested parameters were :

1. Measurement of osteocalcin by sandwich immunoassay.
2. Morphologic examination under the electron-microscope.
3. Calcifying capacity under the electronmicroscope.
4. Cell count under the light microscope.

Assay procedure for the messurement of osteocalcin levels

The assay was a six step-protocol. The first step was the reconstitution of reagents. The "standard" in powder form was reconstituted with 0.25 ml of standard dissolving solution which in itself used as blank. This solution was used to establish a standard curve. The "conjugate" was reconstituted with conjugate dissolving solution. Next, 25 of standard and sample solutions were diluted with 500 of standard dissolving solution and sample diluting solution, respectively. This was followed by the immunological reaction step ; 200 of diluted standard and diluted sample solution were pipetted into glass tubes (12 x 75 mm), then 200 of conjugate solution was dispensed into each tube, after which beads were blotted with filter paper and dropped one by one into each test tube at a constant speed and in order by using forceps. Caution was taken not to dry up the beads. The tube rack was shaken with 20 horizontal strokes, then all the tubes were incubated for 90 minutes in a 37°C water bath. Then, the tubes were washed 5 times with normal saline to remove unreacted components in each test tube by suction with an aspirator. Next, the substrate solution was mixed with an equal volume of chromogen. Four hundred of the mixed solution was pipetted into each test tube and mixed thoroughly, followed by incubation for 30 minutes in a 37°C water bath without shaking. Prior to measurement of the samples, all reaction in each tube was stopped by addition of 1.0 ml of stop solution.

RESULTS

All of the raw data of osteocalcin level was expressed in ng/ml subtracting the background level in the blank media (Table 1). This is to set the control level as 0 ng/ml (Tables 2,3). The sample osteocalcin levels were subsequently converted to ug/ml media (Tables 4,5).

The average osteocalcin levels on days 7,14, and 21 of both the elderly and the younger patients are shown in Tables 6,7. The standard deviation of days 7,14, and 21 are shown in Table 4,5. The cumulative osteocalcin levels are shown in Tables 8,9.

The osteocalcin levels in all samples appeared to be higher than in the control (Table 1). The highest osteocalcin production rate was detected on day 14 and declined on day 21. However, the osteocalcin production rate on day 21 was still higher than day 7. Both elderly and younger patients had similar production rates. When the average osteocalcin levels on days 7, 14, and 21 were compared between the elderly and younger patients on a daily basis, there were no differences as shown in Table 7.

DISCUSSION

Osteoporosis is a disease characterized by a reduction in bone mass that is associated with fractures. A lot of people suffer from osteoporotic fractures every year. The excessive bone loss that characterizes the pathogenesis of osteoporosis results from abnormalities in the bone remodeling cycle. The effort to stimulate bone formation is a new research trend in orthopedics in recent years. The use of osteoblast derived from human bone explants has steadily gained in popularity. Our research is to assess the metabolism of the osteoblast in bone formation.

Table 1. (A and B) Osteocalcin levels in elderly and young patients (ng/ml)

ELDERLY PATIENT (A)

SET NO	1	2	3	4	5	6	CONTROL
DAY 7	3.7	5.4	7.3	4.2	8.7	3.9	1.51
DAY 14	15.8	11.1	12.9	13.1	15.5	18.3	1.49
DAY 21	10.5	9.6	7.7	8.7	11.9	12.3	1.44

YOUNG PATIENT (B)

SET NO	1	2	3	4	5	6	CONTROL
DAY 7	7.2	6.6	4.2	6.2	8.7	5.5	1.5
DAY 14	12.3	13.4	14.5	11.5	20.5	16.4	1.53
DAY 21	11.5	10.1	9.9	10.9	11.3	11.6	1.52

Table 2. Osteocalcin levels of the elderly patient (set control as 0 ng/ml)

SET NO	1	2	3	4	5	6	AVERAGE	S.D.
DAY 7	2.2	3.9	5.8	2.6	7.2	2.4	4	4.69
DAY 14	14.3	9.6	11.4	11.5	14	16.8	12.9	6.21
DAY 21	9.1	8.2	6.3	7.3	10.5	10.9	8.7	4.23

Table 3. Osteocalcin levels of the younger patient (set control as 0 ng/ml)

SET NO	1	2	3	4	5	6	AVERAGE	S.D.
DAY 7	5.7	5.1	2.7	4.7	7.2	4	4.9	3.41
DAY 14	10.8	11.9	12.9	10	18.9	14.8	13.2	7.4
DAY 21	10	8.6	8.4	9.4	9.8	10	9.3	3.15

Table 4. Osteocalcin levels of the elderly patient (concerted to microgram per ml media)

SET NO	1	2	3	4	5	6	AVERAGE	S.D.
DAY 7	4.9	8.7	12.9	5.8	16	5.3	4	4.69
DAY 14	31.7	21.3	25.3	25.5	31.1	37.3	28.7	6.21
DAY 21	20.2	18.2	14	16.2	23.3	24.2	19.3	4.23

Table 5. Osteocalcin levels of the younger patient (concerted to microgram per ml media)

SET NO	1	2	3	4	5	6	AVERAGE	S.D.
DAY 7	12.7	11.3	6	10.4	16	8.9	10.9	3.14
DAY 14	24	26.4	28.7	22.2	42	32.9	29.3	7.4
DAY 21	22.2	19.1	18.7	20.9	21.8	22.2	20.9	3.15

Table 6. Average osteocalcin levels (ng/ml)

	DAY 7	DAY 14	DAY 21
OLDER PATIENT	4	12.9	8.7
YOUNGER PATIENT	4.9	13.2	9.3

Table 7. Average osteocalcin levels (microgram per ml media)

	DAY 7	DAY 14	DAY 21
OLDER PATIENT	8.9	28.7	19.3
YOUNGER PATIENT	10.9	29.3	20.9

Table 8. Average osteocalcin levels in cumulative value (ng/ml)

	DAY 7	DAY 14	DAY 21
OLDER PATIENT	4	16.9	25.6
YOUNGER PATIENT	4.9	18.1	27.4

Table 8. Average osteocalcin levels in cumulative value (microgram per ml media)

	DAY 7	DAY 14	DAY 21
OLDER PATIENT	8.9	37.4	56.9
YOUNGER PATIENT	10.9	40.2	61.1

Osteocalcin, or bone Gla protein, is the most specific marker for osteoblastic activity in bone formation. It is a better marker than either alkaline phosphatase or hydroxyprolene, which have limitations in their specificity[2]. We use sandwich immunoassay as proposed by Honsoda[2] for the measurement of osteocalcin level to overcome the problems of limited region specificity associated with conventional radioimmunoassay. This problem is probably due to the fact that serum osteocalcin consists of heterogeneous molecule, as suggested by Gundberg and Weinstein[10], which has never been specifically detected by conventional methods. The method we employed uses antibodies to the N-and C-terminal regions of human osteocalcin (hOC) that were raised against an N-terminal 20-residue peptide and C terminal 7-residue peptide. This is a direct investigation of osteoblastic metabolism which is free from influences from exogenous factors, such as hormones, metabolites, etc. Thus, it should represent the exact functional status of the osteoblast.

The result of our present study showed that there was osteocalcin released into the media and this confirmed the success of the culture. It also indicated that the osteoblast cell could grow in our media and had normal metabolic function.

Osteocalcin production rates on days 14 and 21 were higher than on day 7, which correlated well with the cell numbers on day 14 and 21. In contrast, we noticed the decline of osteocalcin levels on day 21. This might be due to the fact that on day 21 the amount of important nutrient components was not sufficieat for higher numbers of osteoblast cells. Regarding the average osteocalcin production on days 7, 14, and 21, the result indicated that there was no difference in osteoblastic activity between the elderly (A) and younger patients (B). This result may not be in agreement with the previous research, because age-related decrease in the metabolic activity[11,12,13,14,15] and the

proliferative ability[12,14,16] of osteoblast has been documented. But, regarding the serum osteocalcin level, aging is an increasingly improtant factor[17]. However, the effect of age on osteoblastic function reported in the literature is still in conflict with this report . Our results suggest that, if the osteoblast is grown under the same conditions, there will be similar activity and function regardless of age.

The standard deviation of the three groups on day 14 is the highest. This may suggest that there were technical errors in the testing process, such as inaccurate volumes of the samples and reagents. However, the inaccurate value may be due to the decrease of surviving osteoblasts in some dishes because they could not adhere to the dish bottom, which was the important process for osteoblasts to migrate and differentiate from the donor bones.

This preliminary data suggests that there is no difference in osteoblast metabolism of older and younger patients if they are grown in the same in vitro condition. Our data suggests that there is no difference in osteoblastic metabolism between pre- and post-menopausal women. This study implies that osteoblasts can have normal function if they are grown in the proper environment regardless of age. So it may be applicable in clinical practice where bone grafts from all ages can be utilized without compromising the normal metabolism and function. Because of identical function in both the elderly and younger patient osteoblasts, in this study it may indicate that the decrease of bone mass in osteoporotic patients is not due to a decrease in function of the osteoblast itself, but may be caused by extracellular factors such as hormonal factor, cytokine, etc. So if we can regulate the extracellular factors, we may be able maintain to peak bone mass or stimulate bone formation to treat osteoporosis.

This is a preliminary study, which improves the basic knowledge concerning morphology, metabolism, and function of osteoblasts in Thai people. The greatest benefit from this research is that our osteoblast cell culture technique and laboratory section have been developed in our institute. In the next study, we plan to investigate factors influencing the osteoblastic functions in order to find ways to regulate these functions, to stimulate bone formation in osteoporosis. From this basic cell culture technique, chondrocyte osteoprogenitor, and synovial cells may also be cultured to give us the answers to many orthopaedic problems in the future.

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