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ORIGINAL ARTICLE

Francesco Paolo Cantatore · Addolorata Corrado
Maria Grano · Laura Quarta · Silvia Colucci
Nadia Melillo

Osteocalcin synthesis by human osteoblasts from normal and osteoarthritic bone after vitamin D_3 stimulation

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Abstract Alterations in osteoblast metabolism are involved in the pathogenesis of typical subchondral bone changes in osteoarthritis (OA). Osteocalcin is a specific bone protein, synthesised by the osteoblasts, which can be considered a marker of metabolic activity of these cells. In this study we correlated osteocalcin production from human osteoblasts isolated from healthy and osteoarthritic subjects to the degree of cartilage damage, before and after stimulation with $1,25(OH)_2$-vitamin D_3, the active metabolite of vitamin D_3. We isolated human osteoblasts from cancellous bone of healthy subjects and from subchondral bone of osteoarthritic subjects and considered the osteoblasts corresponding to different degrees of cartilage damage as different cell populations. We determined the osteocalcin production in normal and osteoarthritic osteoblasts from maximal and minimal cartilage damage areas both under basal conditions and after vitamin D_3 stimulation. Compared to normal osteoblasts, under basal conditions osteocalcin production is significantly greater in osteoarthritic osteoblasts, corresponding both to maximal and minimal damage joint areas. No differences were observed between osteoblasts from maximal and minimal damage areas. The response of osteoblasts to vitamin D_3 stimulation appeared to be proportional to the degree of joint damage, as the vitamin D_3-induced increase in osteocalcin is proportionally greater in maximally damaged osteoblasts compared to minimally damaged ones. Thus, after vitamin D_3 stimulation, a significant increase in osteocalcin production by maximally damaged osteoblasts compared to the minimally damaged ones was observed. This study confirms abnormal osteoarthritic osteoblast behaviour and indicates that osteoblasts from different areas of the same affected joint may be metabolically different, supporting the hypothesis that subchondral osteoblasts may play an essential role in the pathogenesis of OA.

Keywords Osteoarthritis · Osteoblasts · Osteocalcin · Subchondral bone · Vitamin D

F. P. Cantatore (✉) · A. Corrado · L. Quarta · N. Melillo
D'Avanzo Hospital, Chair of Rheumatology,
University of Foggia, Via Ascoli,
71100 Foggia, Italy
E-mail: fp.cantatore@unifg.it
Fax: +39-881-733164

M. Grano · S. Colucci
Policlinico Piazza Giulio Cesare,
Department of Human Anatomy,
University of Bari, 74100 Bari, Italy

F. P. Cantatore
Piazza Don Antonio Bello, 2,
Ruvo di Puglia-Bari, Italy

Introduction

Osteoarthritis (OA) is the most common degenerative joint disease, characterised by the progressive degeneration and loss of joint cartilage. OA pathologic changes also occur in other joint components, such as subchondral bone and synovial membrane. In particular, clinical, radiological and biological findings have all demonstrated altered subchondral bone metabolism in OA.

Changes in metabolic activity of subchondral bone cells in osteoarthritis may be consensual to cartilage damage and contribute to disease progression. Some studies indicate that bone alteration may even precede cartilage changes [1, 2], but whether bone sclerosis initiates, or is simply involved in, the progressive cartilage loss is still under discussion. Some observations suggest the existence of an intrinsic change in the bone of osteoarthritic patients, such as an increased expression of growth factors which can determine an increased bone density [3], leading to the hypothesis that OA could be primarily a generalised bone disease which induces an increase in bone stiffness [4].

Subchondral bone is subject to increased remodelling processes which lead to increased stiffness and bone density. The mechanisms responsible for the

pathogenesis of subchondral bone sclerosis in OA are still unclear although several studies indicate that it could be due to abnormal osteoblast behaviour. In some conditions associated with increased bone mineral density, such as osteopetrosis, osteoblasts showed an altered metabolic activity consistent with an increased production of osteocalcin [5]. It has been clearly demonstrated that osteoblast-like cells isolated from subchondral bone of osteoarthritic patients have abnormal metabolic activity [6, 7].

Osteocalcin is a specific bone protein, synthesised almost exclusively by the osteoblast cells and is currently used in clinical practice as a specific biomarker of bone formation. The aim of this study was to determine the changes in osteocalcin production of osteoblast cells derived from osteoarthritic subchondral bone underlying cartilage areas with different degrees of damage.

Patients and methods

Patients and clinical parameters

OA subchondral bone samples were obtained from a total of six patients (two men and four women) aged 60.6 ± 4.8 years (mean ± SD, range: 55–68 years) undergoing total knee joint replacement who were classified as having OA according to the 1986 criteria developed by the American College of Rheumatology [8]. Bone specimens were distinguished depending on the integrity of overlying joint cartilage, thus identifying two distinct areas in which different degrees of macroscopic damage were visible. In the minimal damage areas, joint cartilage was similar to normal cartilage, with a translucent, smooth, intact surface, while in the maximal damage areas the cartilage surface appeared rough and eroded, yellowish, softened, with fibrillation and in some areas was completely absent. The different macroscopic characteristics in the cartilage surface from different damaged areas were related to the different degree of thickening and sclerosis of the corresponding subchondral bone, as confirmed by histological examination. We considered osteoblasts isolated from these two separate damage areas as two distinct cellular populations.

Normal cancellous bone fragments were obtained from six adult subjects (four men and two women) aged 52 ± 10.9 years (mean ± SD, range: 35–61 years) undergoing surgery for traumatic fractures of the distal femoral epiphysis who served as controls. These specimens were selected from the same anatomical regions as the OA specimens, and therefore the comparison between OA and normal osteoblasts was not biased by the anatomical origin of the cell.

Neither patients nor controls were affected by other metabolic bone diseases, and none had received medication, including corticosteroids, which could interfere with bone metabolism for 6 months prior to surgery. Specimens were obtained, and cell cultures prepared, within 8 h of surgery.

Primary cancellous bone osteoblast cell cultures

Cancellous subchondral bone was sampled using a bone biopsy needle in order to obtain very small bone fragments. Each fragment was washed using sterile polysaline buffer solution, then digested with 0.5 mg/ml *Clostridium histolyticum* type I collagenase (Sigma, St. Louis, Mo., USA) with alpha-minimum essential medium (α-MEM, Gibco, Gaithersburg, Md., USA) without serum, supplemented with antibiotics (penicillin 100 UI/ml and streptomycin 100 mg/ml) for 1 h at 37°C to remove all fibroblasts and residual blood cells. Bone chips were subsequently washed in α-MEM supplemented with antibiotics and containing 20% foetal bovine serum (FBS, Gibco, Grand Island, N.Y., USA) and then cultured in sterile culture flasks in the same medium at 37°C in a water-saturated atmosphere containing 5% CO_2. When cells were observed in the flasks, the culture medium was replaced with fresh medium containing 10% FBS every 3 days. Osteoblasts began to grow from the bone specimens approximately 1 week later and proliferated on the flask surface, reaching confluence in 3–4 weeks. At confluence osteoblasts were isolated using trypsin 1% and then transferred into 24-well plates, with 25,000 cells for every plate, until they reached semi-confluence, before performing osteocalcin assay. In order to confirm osteoblastic lineage of adherent cells, we evaluated basal alkaline phosphatase activity in cell lysate by spectrophotometric assay (Metra Biosystem, Mountain View, Calif., USA). Osteocalcin synthesis was evaluated in culture medium by chemiluminescence assay (Nichols Institute Diagnostics, San Juan Capistrano, Calif., USA) for each cellular population (normal osteoblasts, osteoarthritic minimally and maximally damaged osteoblasts) after 48 h under basal conditions and after incubation with 10^{-8}M of 1,25-dihydroxy-vitamin D_3 (vitamin D_3, Roche). Results were normalized per mg/cell protein, measured on cell lysate by the Bradford method (Amresco, Solon, Ohio, USA). Cell lysate was obtained by solubilization in 0.1% sodium dodecyl sulfate (SDS) and osteocalcin production was expressed as ng osteocalcin/mg cell protein, as intracellular total protein content is directly related to a total number of metabolically active cells, as previously described [9]. All experiments were performed at first passage and each of them in triplicate.

Statistical analysis

Results are expressed as mean ± SD. The increase in osteocalcin production after vitamin D_3 stimulation as well as the difference in fold increase of osteocalcin synthesis after vitamin D_3 stimulation were analysed by the Kruskal-Wallis test. The differences in osteocalcin production between the cell cultures were assessed by the Kruskal-Wallis test, using Dunn's test for multiple comparisons. $p < 0.05$ was considered as a significant value.

Table 1 Osteocalcin production in sample of normal osteoblasts. Age and gender of donors did not appear to have any significant influence on osteocalcin production. Triplicates of each sample are indicated, showing the limited variability of results for each experiment. Results are expressed as mean ± SD

Sex	Age	Norm			
		Basal		Vitamin D	
M	42	18.21 23.12 19.12	20.15 ± 2.6	30.01 35.87 33.72	33.2 ± 2.9
F	61	24.08 19.75 23.67	22.5 ± 2.3	41.1 36.06 37.44	38.2 ± 1.8
M	59	20.81 18.05 31.22	23.36 ± 6.9	38.7 37.19 40.81	38.9 ± 1.8
M	35	19.47 22.38 25.95	22.6 ± 3.2	32.12 35.97 39.4	35.83 ± 3.6
M	61	15.81 20.32 10.28	15.47 ± 5	24.03 29.5 22.07	25.2 ± 3.8
F	54	13.98 18.21 10.41	14.2 ± 3.9	21.4 28.08 22.82	24.1 ± 3.5
			19.75 ± 3.9		32.53 ± 6.4

Results

Osteocalcin synthesis was significantly enhanced by vitamin D_3 stimulation in all cellular populations examined ($p < 0.001$), as shown in Table 1 and Table 2. Under baseline conditions normal osteoblasts showed a lower osteocalcin synthesis compared to both maximally and minimally damaged osteoarthritic osteoblasts (*$p < 0.001$, Table 2 and ‡$p < 0.0001$, Fig. 1a). No difference in osteocalcin production was observed between minimally and maximally damaged osteoblasts, although the maximally damaged ones showed a slightly greater osteocalcin synthesis.

After vitamin D_3 stimulation, a lower production of osteocalcin in normal osteoblasts compared to maximally and minimally damaged ones was also observed (**$p < 0.0001$, Table 2 and *$p < 0.0001$, Fig. 1b). Interestingly, enhanced osteocalcin production by vitamin D_3 stimulation is greater in maximally damaged osteoarthritic osteoblasts compared to the minimally damaged and normal ones. As Fig. 2 shows, the "fold increase" of osteocalcin following vitamin D_3 stimulation is significantly higher in maximally damaged osteoblasts than in normal and minimally damaged ones. Thus, maximally damaged osteoblasts showed a greater response to vitamin D_3, exhibiting a statistically significant difference in osteocalcin production compared to minimally damaged osteoarthritic osteoblasts (***$p < 0.0001$, Table 2), which was not observed without vitamin D_3 stimulation.

Discussion

Earlier studies have clearly demonstrated abnormal cellular metabolism in subchondral osteoarthritic bone

Table 2 Osteocalcin production in sample of osteoarthritic osteoblasts. Age and gender of donors did not appear to have any significant influence on osteocalcin production. Triplicates of each sample are indicated, showing the limited variability of results for each experiment. Results are expressed as mean ± SD

Sex	Age	Min				Max			
		Basal		Vitamin D		Basal		Vitamin D	
M	62	62.8 58.73 56.67	59.4 ± 3.1	72.34 75.88 66.85	71.69 ± 4.5	72.8 60.42 59.68	64.3 ± 7.3	180.21 178.44 163.83	174.16 ± 8.98
F	55	51.93 52.99 60.83	55.25 ± 4.8	90.3 84.2 89.59	88.03 ± 3.3	69.2 54.26 56.48	59.98 ± 8.06	191.96 128.14 142.5	154.2 ± 33.48
F	63	65.41 59.87 60.83	62.66 ± 4.9	95.84 96 110.26	100.7 ± 8.2	69.41 75.3 58.09	67.6 ± 8.7	178.34 176.57 142.08	165.33 ± 21.01
F	56	56.21 54.09 64	58.1 ± 5.2	75.03 71.4 73.17	73.2 ± 1.8	56.48 67.08 71.8	65.12 ± 7.8	160.77 144.94 129.71	145.14 ± 15.53
M	68	43.21 40.09 49.6	44.3 ± 4.8	97.91 98.98 104.31	100.4 ± 3.4	48.83 52.5 49.87	50.4 ± 1.9	112.09 137.82 167.69	139.2 ± 27.8
F	60	60.71 58.94 63.92	61.19 ± 2.5	70.4 68.41 88.83	75.88 ± 11.2	62.74 67.7 62.19	64.21 ± 3	153.28 139.76 138.96	144 ± 8.04
			56.81 ± 6.6 *p < 0.0001		84.98 ± 13.3 **p < 0.0001		61.93 ± 6.1 *p < 0.0001		153.67 ± 13.65 **p < 0.0001 ***p < 0.0001

*vs normal osteoblasts under basal conditions; **vs normal osteoblasts after vitamin D stimulation; ***vs minimally damaged osteoblasts after vitamin D stimulation

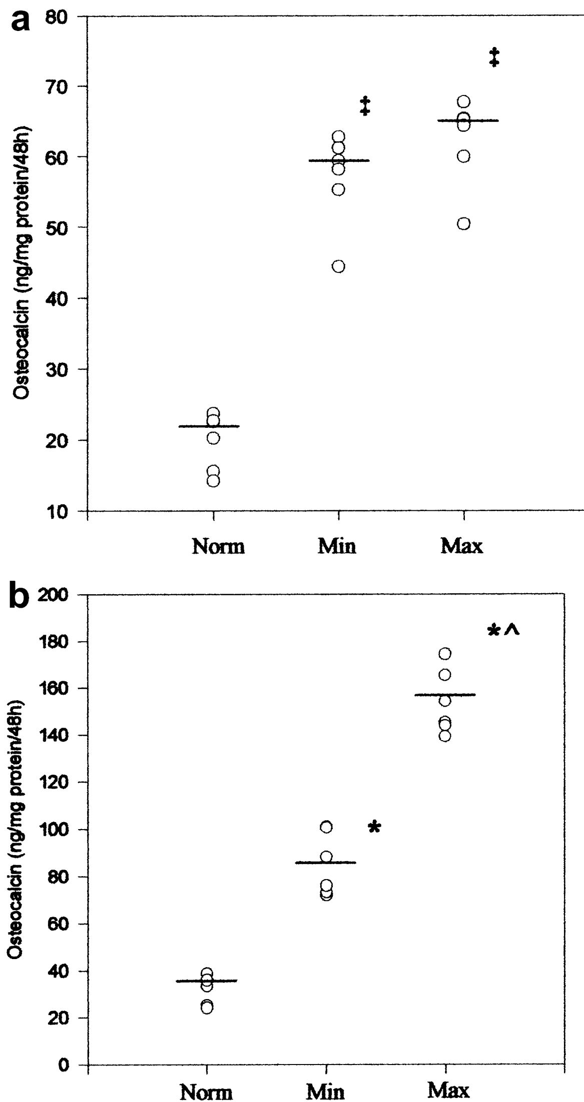

Fig. 1 a, b Osteocalcin production in human osteoblastic cultured cells under basal condition (‡$p < 0.0001$ vs *Norm*) (**a**) and after vitamin D stimulation (*$p < 0.0001$ vs *Norm*, ^$p < 0.0001$ vs *Min*) (**b**). Osteoblasts were obtained from subchondral bone of normal and osteoarthritic subjects, and we considered the cells isolated from osteoarthritic joints as two distinct cellular populations, depending on the different damage degrees of corresponding cartilage. Osteocalcin production is expressed as ng/mg intracellular protein/48 h. Results are shown as scatter plots, with *circles* representing the result from each experiment; *bars* indicate the median of osteocalcin production in each cell population. For each osteoblast culture the osteocalcin measurement was performed in triplicate. *Max* maximally damaged osteoblasts, *Min* minimally damaged osteoblasts, *Norm* normal osteoblasts

[6, 10, 11, 12]. Dieppe et al. [13] observed that increased bone remodelling correlated with the severity of OA, and increased osteocalcin production in osteoarthritic osteoblasts has been observed by other authors [6], but a correlation between disease severity and both osteocalcin synthesis and increased response to vitamin D_3 stimulation has never been demonstrated, although their exact significance in the pathogenesis of this disease is unclear. In this preliminary report, we demonstrate that

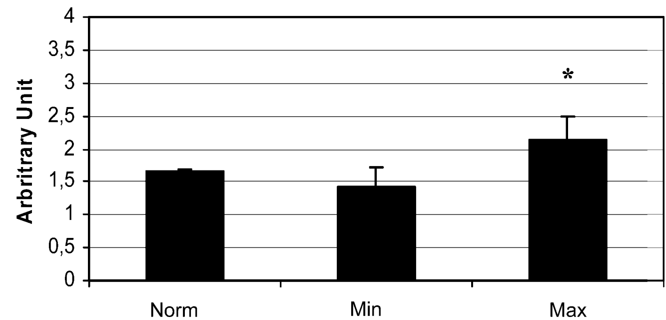

Fig. 2 Comparative increase in osteocalcin synthesis after vitamin D_3 stimulation between normal and osteoarthritic osteoblasts (*$p < 0.001$ vs *Min* and *Norm*). Results are expressed as mean ± SD of ratios between osteocalcin production after vitamin D_3 stimulation and under basal conditions. *Norm* normal osteoblasts, *Max* maximally damaged osteoarthritic osteoblasts, *Min* minimally damaged osteoarthritic osteoblasts

osteoblasts of osteoarthritic subjects produce a greater quantity of osteocalcin compared to those of normal subjects. Moreover, the vitamin D_3-induced increase of osteocalcin in osteoblasts from maximally damaged articular areas is proportionally greater than in normal and minimally damaged osteoarthritic osteoblasts, as shown in Fig. 2. The greater response to vitamin D of osteocalcin production in osteoarthritic osteoblasts could sustain the hypothesis of a metabolic activation of those cells proportional to anatomic damage. A greater osteocalcin production by OA osteoblasts appears to be related to the extent of the damage in overlying articular cartilage, as can be seen in the difference in osteocalcin production of maximally and minimally damaged osteoblasts of the same subject after vitamin D stimulation, even if under basal conditions this is not significant (Table 2).

Differences in osteocalcin production in response to $1,25(OH)_2D_3$ in osteoblastic cells depending on different skeletal site of origin have previously been shown [14], but the specimens that we used as normal controls were selected from the same anatomical regions as the OA specimens, and therefore the comparison between OA and normal osteoblasts was not biased by the anatomical site of cell origin. Increased bone turnover and related bone markers following traumatic fracture have been observed [15], but this is seen between the 2nd and 4th week after a traumatic event, while in our study the control group's osteoblasts were isolated between 1 and 3 days after the traumatic event.

Differences in the production of various osteoblastic markers, such as osteocalcin, alkaline phosphatase and C-terminal type I procollagen, depending on gender and donor age, have been observed in human osteoblasts as well as in the hormonal responsiveness of these cells, but the available data are very discordant [14, 16, 17]. Many authors have demonstrated that both serum circulating osteocalcin and in vitro osteocalcin production from human osteoblastic cells appeared to decrease with age [18, 19], but others have found no differences between

osteocalcin production by cultured osteoblast from older and younger subjects [14]. Although the limited number of subjects per group in our study does not permit generalised conclusions, it would seem that donor age does not influence osteocalcin production in the relative osteoblastic cultures, as shown in Table 1 and Table 2.

Various authors have previously demonstrated the influence of sex on serum levels of osteocalcin. A different metabolic activity in osteoblasts from male and female subjects has also been shown, and it seems that osteocalcin production in both sexes has a different pattern depending on age. The data available to date, both in vivo and in vitro, seem to indicate the tendency for lower osteocalcin production in females, even if in this case data are also discordant. However, in our study the group of osteoarthritic subjects from whom we isolated the osteoblasts which produced more osteocalcin were prevalently female. These data, in direct contrast to the majority of existing literature, further confirm the influence of the osteoarthritic pathologic process on bone cell metabolism rather than on sex.

That subchondral bone changes are consequent, concomitant, or even precede the typical cartilage alteration in OA is still a matter for debate. Westacott et al. have observed that osteoblasts derived from subchondral bone of osteoarthritic subjects can alter cartilage metabolism in vitro, exhibiting an increase in degradation processes, and osteoblasts derived from different joint regions, corresponding to a different mechanical load, showed a different capacity to modify the metabolic cartilage activity [20]. These data further confirm that metabolic changes in subchondral OA bone can vary depending on the degree of joint damage and can affect cartilage metabolism in different ways. Important evidence which supports the pathogenic importance of bone in determining cartilage alteration during OA is the observation that osteopetrosis, a rare inherited disorder characterised by abnormal bone sclerosis, is associated with a high risk of OA [21]. Conversely, there are several epidemiological studies that suggest the existence of an inverse relationship between OA and osteoporosis [22, 23], although an in vivo study showed that an increase in serum osteocalcin induced by $1,25(OH)_2D_3$ was similar in osteoarthritic and osteoporotic subjects [24]. This supports the hypothesis that primary OA implies widespread morphological and biochemical changes in bone and could initially be a bone disease. In generalised osteoarthritis, an increased osteocalcin production was observed in bone from the iliac crest, which is a site that is not affected by mechanical or degenerative changes [3].

Abnormal OA osteoblast behaviour is involved in the genesis of bone sclerosis and osteophyte formation, and osteoblasts from different areas of the same affected joint may be metabolically different and probably contribute to pathogenic events that lead to degradation of overlying cartilage. A very interesting hypothesis is the possibility of an interaction between bone and cartilage as an essential part of the disease, leading or contributing to cartilage destruction, but further studies are needed to demonstrate whether subchondral osteoblast hyperactivity in OA precedes, or depends on, cartilage degeneration.

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