Osteocyte density changes in aging and osteoporosis

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Osteocyte Density Changes in Aging and Osteoporosis

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Recently, it was suggested that osteocytes are involved in the regulation of bone remodeling. We have examined human trabecular bone of the iliac crest of fracture patients and control subjects to determine if osteoporosis is associated with changes in osteocyte density or osteocyte death. The relationships of these parameters with age was also investigated. It was found that osteocyte death was not related to age, nor was it increased in osteoporosis compared with the controls. In healthy adults ranging from 30 to 91 years, lacunar number per bone area decreases with advancing age, from about 210/mm² to 150/mm². Significantly higher lacunar and osteocyte numbers per bone tissue volume were found in osteoporotics than in controls (17,100 lacunae/mm³ and 13,300 osteocytes/mm³ vs. 12,900 lacunae/mm³ and 10,500 osteocytes/mm³, respectively), whereas lacunar area was significantly reduced in osteoporotics (from 44.1 μ m³ to 39.1 μ m²). These findings are compatible with the hypothesis that, in osteoporosis, osteoblasts produce less bone per cell. This can in turn explain the reduced wall thickness, which has previously been described as characteristic for osteoporosis. (*Bone* 18:109–113; 1996)

Key Words: Osteocyte; Trabecular bone; Osteocyte lacunar number; Osteocyte lacunar size; Osteoporosis.

Introduction

Bone is a dynamic tissue undergoing continuous renewal. The mechanical integrity of bone is ensured by removal of bone and subsequent replacement by new bone. After the age of 25–30 years, a slightly negative balance between bone resorption and formation may cause progressive bone loss. Usually, mechanical integrity is maintained. However, in osteoporosis, excessive bone loss and loss of structural elements can lead to mechanical failure.²⁴ As turnover is highest in cancellous bone, osteoporosis becomes manifest particularly in regions where the trabecular architecture is of great structural importance such as, for instance, in the vertebral body and the femoral trochanter. The cause of osteoporosis is sought in a disturbed regulation of the bone remodeling process.⁸ It is well established that mechanical usage is essential for the maintenance of bone. Hence, several investigators suggested that osteoporosis is possibly caused by an inadequate appraisal of the mechanical load in bone, because the changes in bone occurring in osteoporosis are similar to changes

resulting from disuse.^{10,25} Yet, the normal regulation of bone remodeling has still to be unraveled. The regulation of the remodeling sequence and the coupling mechanism between the activity of osteoclasts and the activation of osteoblasts are still unknown.

Recently, it was suggested that osteocytes play a role in the regulation of bone remodeling. It was hypothesized that osteocytes regulate the recruitment of basic multicellular units (BMUs) in response to mechanical stimuli,^{1,3,15,17} and that they play a role in the modulation of osteoblast activity and the recruitment of osteoblasts which differentiate into osteocytes.¹⁸ A number of experiments (in vivo and in vitro) showed that osteocytes respond to mechanical loading with an increased production of factors which are known to affect bone turnover.^{14,15} Mullender et al.²⁰ and Mullender and Huiskes,¹⁹ using a computer simulation model, have shown that trabecular modeling patterns due to mechanical stimuli can be explained quantitatively by assuming osteocytes to act as strain-sensing cells in a regulatory process. They showed that osteocyte density and range of influence, i.e., the distance from which they can affect BMU activity, may have distinct effects on the trabecular morphology. They also showed that a reduced sensitivity of osteocytes to mechanical load caused bone loss in a similar way as did disuse.

The above hypotheses and findings have led us to the hypothesis that a disturbance in the regulation of bone remodeling in osteoporosis may be associated with a lack of osteocytes or inefficacy of function. To investigate this hypothesis, we have determined the number of osteocytes per bone area and volume and the fraction of empty lacunae, as an indicator of osteocyte death, in osteoporotic patients and control subjects.

Materials and Methods

Subjects

The osteoporosis group consisted of 14 patients (4 men and 10 women, age 65.2 $\pm$ 7.2 years [mean $\pm$ SD]) with each at least one vertebral crush fracture (collapsed vertebra), and 23 patients (9 men and 14 women, age 73.6 $\pm$ 11.3) with hip fractures. Transiliac biopsies were taken from all patients. Iliac crest bone samples from 25 autopsy subjects (sudden death in previously healthy persons) and 4 patients who received cosmetic or orthopedic surgery unrelated to bone disease were obtained for the control group (24 men and 5 females, age 57.0 $\pm$ 18.5 years). None of the control group had a history of any disease known to predispose to osteoporosis, and neither control subjects or patients received drugs with known effects on bone.

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Histology

The undecalcified biopsies were embedded in methylmetacrylate, sectioned (5 μm) and stained with Goldner's trichrome. Histomorphometry was performed on the trabecular bone of two sections per biopsy. Microscopic fields were sampled in equally spaced rows by moving the specimens in equally sized steps such that the total specimen area was covered. A Zeiss integrating eyepiece was used for the measurement of trabecular bone volume (BV/TV) by counting the number of hits and the number of intersections. More extensive histomorphometric data of both groups were published in a different study.³¹

In addition, the number of lacunae occupied by osteocytes and the number of empty lacunae per bone area were measured in trabecular bone. Osteocytes are colored red and are readily visible in the bone matrix. Empty lacunae were defined as lacunae without any visible remnant of a cell inside. These measurements were performed using a Zeiss microscope in conjunction with a digital image analysis system (Videoplan). Twenty fields were sampled in two sections per subject ($\times 25$ objective) by moving the specimen in equally sized steps in x and y directions. In this way, a total bone area of approximately 1 mm^2 per subject was measured to determine lacunar and osteocyte numbers. The investigator had no knowledge about the origin of the sections. From these measurements, the following parameters were deduced: the fraction of empty lacunae (number of empty lacunae/total number of lacunae); the total number of lacunae per bone area (N.Lc/B.Ar); and the number of lacunae occupied by osteocytes per bone area (N.Ot/B.Ar).

The measured number of osteocyte lacunae per area depends on both the number of lacunae per volume and the average lacunar size. Because we wanted to determine if differences in lacunar number per bone area between controls and osteoporotics are due to differences in lacunar size, or to differences in lacunar number per bone volume, we also measured lacunar area (Lc.Ar) as an indicator of lacunar size. These measurements were made by outlining at least 75 lacunae per section in randomly selected fields, totaling at least 150 lacunae per subject ($\times 100$ oil immersion objective).

The measured parameters N.Ot/B.Ar, N.Lc/B.Ar, and Lc.Ar were used to estimate the number of osteocytes per bone volume (N.Ot/BV) and the number of lacunae per bone volume (N.Lc/BV). First, the measured lacunar area was used to calculate an average "osteocyte radius," R . Assuming that osteocytes have a spherical shape, the measured Lc.Ar is equal to $\text{Lc.Ar} = \frac{2}{3} \pi R^2$, for infinitely thin sections. If the equation is corrected for the section thickness (t), and if it is further assumed that k is the thickness of the smallest part of a cell which must be included in the section for its identification, the equation is modified to:

$$\text{Lc.Ar} = \pi \left(R^2 - \frac{(R - k)^3}{3(R - k + \frac{1}{2}t)} \right). \quad (1)$$

As this equation is not easily inverted to calculate R , it was estimated by fitting R to obtain the measured Lc.Ar with a maximal error of 0.005 μm^2 . This is only a rough estimate of R , because osteocytes in fact have an elliptical shape.

The number of osteocytes and lacunae per volume were calculated according to:

$$\text{N.Ot/BV} = \frac{\text{N.Ot/B.Ar}}{2R + t - 2k}, \quad (2)$$

$$\text{N.Lc/BV} = \frac{\text{N.Lc/B.Ar}}{2R + t - 2k}, \quad (3)$$

where k and t are the same factors as above.²⁷ A section thickness of $t = 5.0 \mu\text{m}$ and a value of $k = 0.2 \mu\text{m}$ was used.

Statistical Analysis

Averages and standard deviations of all measured parameters were determined per group. The reproducibility of the method for measuring lacunar density and size was assessed using the coefficient of variation. These parameters were measured four times in six sections by one observer and were again measured by a second investigator. To increase reproducibility, all measurements were conducted by the same investigator. Correlations between parameters and age were examined by linear regression analyses. Differences between groups were tested using the two-tailed Student's t -test. To exclude effects of age differences, only subjects older than 55 years were used for the comparison between the control group and the osteoporosis group. However, it should be noted that the group of hip fracture patients was still older than the control group.

Results

The values for reproducibility are given in **Table 1**. The mean values and standard deviations of all measured parameters are given in **Table 2**. No differences in parameter values were found between males and females within each group. BV/TV was significantly decreased in the osteoporotic patients relative to the control group older than 55 years. The BV/TV of two control subjects could not be reliably measured because the specimen sizes were too small. The N.Lc/B.Ar and also the N.Ot/B.Ar were significantly higher in younger controls than in older controls. In addition, the N.Lc/B.Ar and N.Ot/B.Ar were significantly higher in the hip fracture and vertebral fracture groups relative to the older control group (**Figure 1**). Lc.Ar was significantly smaller in hip fracture patients and in the combined osteoporosis group than in the older control subjects (**Figure 2**). As the lacunar size was smaller in the osteoporosis group relative to the older control group, the differences in number of lacunae and osteocytes per bone volume were even more pronounced than the differences between numbers per bone area. The N.Lc/BV and N.Ot/BV were also significantly higher in the younger controls in comparison with the older controls. The fraction of empty lacunae did not differ significantly between groups.

The relationships between several parameters and age are presented in **Table 3**. In the control group, the number of lacunae and osteocytes per bone area and per bone volume declined significantly with advancing age (**Figure 1a** and **b**). The fraction of empty lacunae and lacunar area were not significantly related to age. No significant relationships between age and other parameters were found in the osteoporosis group.

Table 1. Coefficient of variation (%) for repeated measurements^a

	Intraobserver	Interobserver
Lacunae per bone area	4.5	3.5
Osteocytes per bone area	4.8	5.3
Percentage of empty lacunae	3.9	7.6
Lacunar area	14.6	23.1

^a Reproducibility of lacunar numbers, numbers of osteocytes, and fraction of empty lacunae are good, but measurements of osteocyte size show large variation.

Table 2. Subject data and histomorphometric parameters in control subjects and osteoporotic patients (average values and standard deviations)

	Controls			Osteoporotic patients >55 years		
	≤55 years	>55 years	Combined	Hip fract.	Vert. fract.	Combined
Total	12	17	29	22	12	34
Male	12	12	24	8	2	10
Female	—	5	5	14	10	24
Age (years)	39.0 ± 7.7 ^b	69.7 ± 12.0	57.0 ± 18.5	79.7 ± 9.3 ^a	67.6 ± 4.7	75.4 ± 9.9
BV/TV (%)	18.0 ± 6.3	16.8 ± 5.9 ^c	17.4 ± 6.1	12.5 ± 4.1 ^a	8.4 ± 4.2 ^b	11.0 ± 4.5 ^b
N.Lc/B.Ar (mm ⁻²)	206.5 ± 29.2 ^a	165.7 ± 38.3	182.6 ± 39.9	203.0 ± 29.7 ^a	228.9 ± 28.2 ^b	212.1 ± 31.4 ^b
N.Ot/B.Ar (mm ⁻²)	172.8 ± 34.9 ^a	135.1 ± 38.0	150.7 ± 40.7	158.3 ± 23.6 ^a	176.0 ± 21.6 ^a	164.5 ± 24.2 ^a
Fract. empty lacunae	0.17 ± 0.08	0.19 ± 0.10	0.18 ± 0.09	0.22 ± 0.05	0.22 ± 0.06	0.22 ± 0.05
Lc.Ar (μm ²)	47.3 ± 5.8	44.1 ± 7.3	45.5 ± 6.8	38.5 ± 4.1 ^b	40.0 ± 6.2	39.1 ± 4.9 ^b
N.Lc/BV (10 ³ mm ⁻³)	15.6 ± 2.0 ^a	12.9 ± 3.2	14.0 ± 3.0	16.5 ± 2.5 ^b	18.4 ± 2.2	17.1 ± 2.5 ^b
N.Ot/BV (10 ³ mm ⁻³)	13.1 ± 2.4	10.5 ± 3.0	11.6 ± 3.0	12.8 ± 1.9 ^b	13.7 ± 2.2 ^a	13.3 ± 2.0 ^b

^a Significantly different from the control group >55 years ($p < 0.05$).

^b Significantly different from the control group >55 years ($p < 0.01$).

^c Measurements of two subjects discarded ($n = 15$).

Discussion

Recently, it was suggested that osteocytes are involved in the regulation of bone remodeling.^{1,3,15,17–20} Furthermore, it was proposed that the incorporation of osteoblasts into the matrix (i.e., the inclusion of osteocytes) is a highly regulated process in which the preosteocytes themselves and osteocytes already incorporated are actively involved.^{17,21–23} Therefore, it is plausible that changes or disturbances in (pre-)osteocyte function also affect osteocyte morphology and osteocyte number. If we assume that osteocytes do indeed play a central role in the regulation of bone turnover, a disturbance of this regulatory process may be caused by disturbances in osteocyte presence or viability. The questions investigated in this study were: Does osteocyte number and size differ between osteoporotic patients compared with controls, and does excessive osteocyte death occur in osteoporosis? In addition, we have investigated whether osteocyte death, osteocyte density, and lacunar density are related to age.

Few investigators have actually measured osteocyte density. Hobdell and Howe¹² found that the average volume of bone

matrix associated with one osteocyte lacunae was 0.000077 mm³ in human adult lamellar bone. This is equivalent to 13,000 mm³ lacunae per bone volume. Sissons and O'Connor²⁷ report values for N.Lc/BV between 13,900 and 19,400 mm⁻³ in human cortical bone, depending on the type of sections used. The most accurate method to determine numbers of cells per volume is known as the disector method.^{11,30} However, this method requires serial sectioning of the specimen. As our specimens were already processed for the purpose of histomorphometric analysis, it was not possible to use the disector method. Instead, we measured lacunar area which is a direct estimate of lacunar volume and the number of osteocytes per bone area, which is a method generally accepted for other cell types such as osteoblasts or osteoclasts. Although our calculated figures for osteocyte and lacunar numbers per bone volume only give an estimate of the actual figures, our figures (N.Lc/BV ranging from 12,900 to 18,400 mm⁻³) agree very well with the values reported earlier.

Significant differences were found in osteocyte density and lacunar density between osteoporotics and controls (55 years). Lacunar and osteocyte number per bone area were significantly

Figure 1. The relationships between age and the number of lacunae per bone area (a) and the number of osteocytes per bone area (b). In the control group (■), the number of lacunae as well as the number of osteocytes per bone area decrease with age. In both groups of fracture patients (vert. fract., □; hip fract., ⊠), the numbers of lacunae and osteocytes per bone area are significantly increased compared with controls of similar ages.

Table 3. Linear regression equations and correlation coefficients of several parameters in relation to age

x	y	Group	n	Regression equation	Correlation coefficient
Age (years)	N.Lc/BAr (mm^{-2})	Controls	29	$y = 241.32 - 1.03x^b$	-0.48^b
		Osteoporotics	37	$y = 267.65 - 0.76x$	-0.27
Age (years)	N.Ot/BAr (mm^2)	Controls	29	$y = 210.10 - 1.04x^b$	-0.47^b
		Osteoporotics	37	$y = 200.30 - 0.49x$	-0.23
Age (years)	N.Lc/BV (10^3 mm^{-3})	Controls	29	$y = 18.00 - 0.07x^a$	-0.42^a
		Osteoporotics	37	$y = 21.16 - 0.06x$	-0.25
Age (years)	N.Ot/BV (10^3 mm^{-3})	Controls	29	$y = 15.66 - 0.07x^a$	-0.44^a
		Osteoporotics	37	$y = 15.83 - 0.04x$	-0.20

^a $p < 0.05$; ^b $p < 0.01$.

increased in osteoporotic patients relative to controls. A higher number of osteocyte lacunae per bone area may be due to a higher number of lacunae per bone volume and/or to enlarged lacunar sizes.^{27,30} Enlarged osteocyte lacunae have been reported in osteoporotic patients³⁴ and in calcium-deficient rats.^{28,29} However, in this study we found that the lacunar area was smaller in the osteoporosis group compared with controls. Hence, the differences in numbers of lacunae and osteocytes per bone volume were even more pronounced than these differences in numbers per bone area. These results suggest that, in osteoporosis, less bone volume was produced per osteocyte. There are three possible explanations for this phenomenon: (1) a higher percentage of the bone forming osteoblasts is embedded as osteocytes, whereas the average activity or longevity is unchanged; (2) the bone forming activity of osteoblasts is reduced; and (3) the average life-span of osteoblasts is shorter. The latter two explanations seem to be the most likely ones, because they are compatible with the findings that bone formation and mean wall thickness are decreased in osteoporotic patients compared with normals.^{4,6} Eriksen and Kassem⁷ even state that the most marked difference between osteoporotic and normal women is a considerable reduction of mean wall thickness in osteoporotics. While Eriksen and Kassem⁷ suggested this to be due to a reduced osteoblastic vigor, Lips et al.¹⁶ explained the decrease in mean

wall thickness by a decreased longevity of the osteoblasts. To distinguish between these two hypotheses, Shih et al.²⁶ investigated the relationship between bone formation rate and osteoblast surface. They found that, although bone formation rate is reduced in women with osteoporosis compared to normal women, the relationship was similar in both groups. This implies that, in osteoporotics, either fewer osteoblasts are recruited or that active osteoblasts are transformed quicker into less active ones. Our results suggest that the bone forming capacity per cell is reduced, which is compatible with the latter explanation of Shih et al.²⁶ Furthermore, a decreased longevity of osteoblasts may explain that more are incorporated as osteocytes per bone volume. The reduced lacunar size may also indicate a history of reduced activity of these cells.

The relationship between age and osteocyte density in humans has (to our knowledge) not yet been reported. A significant decrease of osteocyte and lacunar density associated with a (not significant) decrease in lacunar size was found with increasing age in healthy adults from 30 to 91 years of age. This decrease was also observed in the osteoporosis group, but it was not significant due to the smaller age range. Some controversy exists in these results. As Lips et al.¹⁶ showed that mean wall thickness in trabecular bone decreases with aging, it would be expected that osteocyte number increases with increasing age and that this increase is more pronounced in osteoporosis. However, it was found that osteocyte density decreases with age. Hence, it seems that two different phenomena occur at the same time. To explain these phenomena, it is necessary to investigate the relationship between osteocyte number and remodeling activity more closely.

The percentage of empty lacunae was used as an indicator of osteocyte death. Nonviable osteocytes can stain normally up to 16 weeks,¹³ but a gradual loss of osteocytes will be reflected by an increase of empty lacunae. It is possible that lacunae appear to be empty due to sectioning artifacts. However, this is very unlikely because the bone is undecalcified and the cellular processes of osteocytes are integrated within the bone matrix. Furthermore, if artifacts occur, it is assumed that they occur equally in both control and osteoporosis groups such that differences in the number of empty lacunae between the two groups will still be detected. The percentage of empty lacunae varied from 5% to 40% in all subjects. No significant correlation was found between the percentage of empty lacunae and age in both the control and the osteoporosis groups. Similar results were found by Baud and Aul² who looked at bone from the mandible. In contrast, Wong et al.^{32,33} observed a loss of viable osteocytes in the femoral head with increasing age and Frost⁹ also found that the percentage empty lacunae increased with age. Dunstan et al.⁵ showed that the occurrence of osteocyte death with age depends strongly on the location of measurement. They found that osteocyte death did increase with age in the femoral head but did not

Figure 2. The lacunar area is given as a function of age. Although the regressions are not significant, they illustrate that the lacunar area is reduced in osteoporosis compared with the control group (control, ■; vert. fract., □; hip fract., ×). Regressions (---) controls $y = 49.96 - 0.08x$ ($p = 0.26$); and (.....) osteoporotics $y = 43.11 - 0.05x$ ($p = 0.46$).

increase in the second lumbar vertebrae. They suggested that bone with a constant high viability is remodeled at a higher rate, and thus bone is replaced before the osteocytes have a chance to die.

The fraction of empty lacunae was not significantly elevated in osteoporosis compared with controls. Hence, it seems that osteoporosis is not associated with increased osteocyte death, which challenges the hypothesis that mechanical load is appraised inadequately due to increased osteocyte death. However, the hypothesis that osteocytes are less sensitive to mechanical stimuli still needs to be investigated.

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