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Osteocytes in Chronic Uremia

Differential Count of Osteocytes in Human Femoral Bone

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Summary. Differential counts of osteocytes were performed in human cortical femoral bone of 40 patients with chronic renal failure and of 40 patients without skeletal disease. In undecalcified ground sections (50–70 μ) stained with basic fuchsine, osteocytes were differentiated into small (= neutral) osteocytes, enlarged (= metabolically activated) osteocytes and empty lacunae (= dead osteocytes). In uremia the fraction of activated cells and of empty lacunae is markedly increased, whereas the fraction of small neutral osteocytes is reduced. These findings are somewhat more pronounced in Haversian than in interstitial bone. The activated osteocytes are randomly distributed in Haversian systems and do not accumulate in the outer and older parts of individual osteones of uremic or nonuremic subjects. These findings are presumably caused by elevated serum parathyroid hormone levels, which lead to increased activation of osteocytes. Activation of osteocytes may induce shortening of osteocyte survival time.

Zusammenfassung. In der Corticalis der Femurdiaphyse wurden bei 40 Patienten mit chronischer Niereninsuffizienz und bei 40 skeletgesunden Kontrollpersonen Osteocytendifferentialzählungen durchgeführt. An unentkalkten Knochendünnschliffen wurde nach Färbung mit basischem Fuchsin der Anteil der kleinen neutralen Osteocyten, der großen metabolisch aktivierten Osteocyten und der Anteil der leeren Osteocytenlakunen ausgezählt. In der Corticalis steigt bei chronischer Niereninsuffizienz die Zahl der großen aktivierten Osteocyten und der leeren Lakunen signifikant auf Kosten der kleinen Osteocyten an. Die Veränderungen sind in Haversschen Osteonen etwas stärker ausgeprägt als in interstitiellen Knochenlamellen.

Aktivierte Zellformen sind in den äußeren älteren Abschnitten der Osteone nicht häufiger anzutreffen als in der Nähe des Gefäßkanals. Die Befunde werden auf eine vermehrte Aktivierung der Osteocyten unter dem Einfluß erhöhter Serum-Parathormonspiegel zurückgeführt. Die Zunahme aktivierter Zellen und leerer Osteocytenlakunen deutet auf eine Verkürzung der Osteocytenlebenszeit bei sekundärem Hyperparathyreoidismus hin. Die gesteigerte metabolische Aktivität der Osteocyten in der Urämie ist angesichts der urämischen Störung des Calciumstoffwechsels mit Hypocalcämie bemerkenswert.

Since serum calcium homeostasis has recently been shown to depend primarily on osteocytic activity (Neuman and Ramp, 1971), the morphology of osteocytes has attracted considerable interest (Baud, 1962, 1968; Vittali, 1968; Mathews and Martin, 1971). The population of osteocytes cannot be considered as a homogeneous group of cells. On the contrary, they may be in rather different functional

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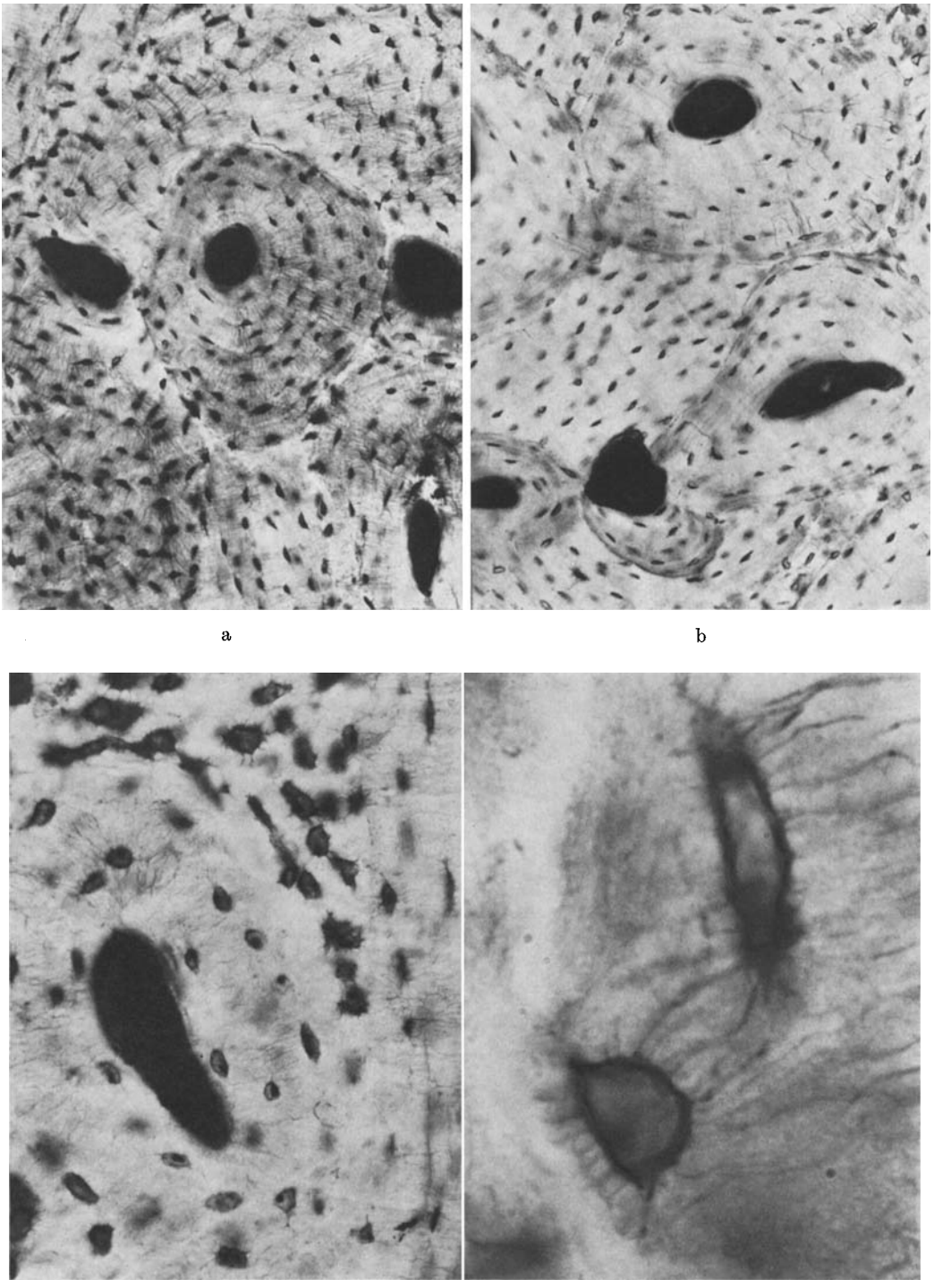

a b

c d

Fig. 1a—d

states, which are also reflected by differences in morphology (Jande, 1971, 1972). Based on electron microscopic studies, the submicroscopic structure of osteocytes with their surrounding matrix has been elucidated. According to Baud (1968) the processes of osteolysis and osteoplasia can be recognized and are evidence of the role of osteocytes in the hormonal control of calcium homeostasis.

By light microscopy Baud and Auil (1971) were able to differentiate small osteocytes, large osteocytes and empty lacunae. Small osteocytes are apparently in a neutral state and large osteocytes in an activated state with osteolysis or osteoplasia, whereas empty lacunae are evidence of cell death. A characteristic histogramm of these different osteocytes has been obtained in normal human bone (Baud and Auil, 1971). Although activated osteocytes have been described decades ago in various metabolic osteopathies, such as rickets or hyperparathyroidism (v. Recklinghausen, 1910; Jaffe and Bodansky, 1930), no systematic quantitative investigations have been carried out. In our experience activation of osteocytes is a highly sensitive morphologic sign of parathyroid hormone action on the skeleton. Likewise activation of osteocytes is the earliest morphological change that is detectable in the bone after the experimental administration of parathyroid hormone (Jande, 1972).

In view of the important role of osteocytes in the disturbance of calcium metabolism in renal insufficiency, differential counts of osteocytes were obtained in cortical bone of uremic patients.

Material and Methods

Circular disks (1 cm thick) were taken from the proximal diaphysis of the right femur in 40 patients with chronic renal insufficiency of different aetiology (20 males, 20 females; serum creatinine >5 mg-%, mean age $38{,}4 \pm 19.8$ y) and in 40 age and sex matched controls without skeletal disease. The samples were obtained at autopsy 5–12 hours after clinical death and fixed immediately in 70% cold alcohol. Ground sections of 50–70 μ thickness were prepared from the anterior medial quadrant. The sections were dehydrated with alcohol, stained with 5% basic fuchsine (10 min) and mounted on slides. Osteocyte differential counts (differentiating small osteocytes, enlarged osteocytes and empty lacunae) were performed (using oil immersion, enlargement 1:1000) according to the criteria given by Baud and Auil (1971): The small osteocyte is located in a flattened ellipsoid lacuna with a smooth and regular outline, the short axis not exceeding 4 μ: It pocesses a dense and homogeneously stained nucleus and little cytoplasma. The enlarged osteocyte is found in an irregular oval lacuna with a small axis exceeding 4 μ. The nucleus is swollen and shows coarse granules of fuchsinophilic chromatin. It is enclosed by broad cytoplasmic rims. Empty lacunae have the same outline as enlarged lacunae but contain no material that can be identified as part of

Fig. 1a—d. Femoral cortical bone. Undecalcified ground sections (50 μ). Basic fuchsin stain. Microphotographs. a 32 year old man, chronic renal failure. Numerous enlarged osteocytes in interstitial and in Haversian bone. Increased fuchsin permeability of bone indicating low mineral density. Enl. 1:150. b 38 year old man, no skeletal disease. The fraction of enlarged osteocytes is marked smaller than in chronic uremia. Fuchsin permeability of bone is less extensive. Enl. 1:150. c Uremic osteopathy with enlarged osteocytes, irregularly shaped lacunae and rough lacunae outline. Cellular activation is more pronounced in interstitial (above) than in Haversian bone. Enl. 1:325. d Empty osteocytic lacunae: enlarged cavities with rough border containing no osteocytes. Enl. 1:1000 (oil immersion). a–c Reduced to $^5/_7$

a living osteocyte (Fig. 1a—d). In all cases 500 osteocytes from Haversian osteones as well as from interstitial lamellae were counted by two examiners without knowledge of the clinical history. The topographic distribution of enlarged osteocytes in individual Haversian systems was evaluated by counting the percentage of large osteocytes both in the inner and in the outer part of the Haversian system (the section of the radius from the outer edge of the central canal to the cement line was halved and osteocytes in the respective circular rings were counted). Statistical analysis of the results was carried out by students t test. All data are given as mean $\pm$ standard deviation.

Results

The results of the differential counts are summarized in Table 1 and represented graphically in Fig. 2a and b.

a) Osteocyte Differential Count in Haversian Bone (Fig. 2a)

In Haversian lamellar bone of healthy subjects without skeletal disease the number of small neutral osteocytes was highest. The number of large activated osteocytes was markedly smaller, whereas empty lacunae were rather infrequent. In contrast, in patients with chronic renal insufficiency the number of large activated osteocytes as well as the number of empty lacunae was markedly increased, whereas the fraction of small osteocytes was clearly decreased.

b) Osteocyte Differential Count in Interstitial Bone (Fig. 2b)

In patients without skeletal disease the fraction of activated large osteocytes in interstitial bone is much higher than the fraction of small neutral cells. This contrasts with what is seen in Haversian bone (Fig. 2a). The fraction of empty lacunae is small but still exceeds that of empty lacunae in Haversian bone. In uremia the number of activated osteocytes and of empty lacunae increases even more, whereas the fraction of small osteocytes decreases. All differences are highly significant.

c) Topographical Distribution of Enlarged Osteocytes within Individual Osteones

Both in patients without skeletal disease and in patients with uremia the fraction of enlarged osteocytes in the inner part of the Haversian systems (definition s. material and methods) was approximately 25% of the total number of enlarged osteocytes/osteone (control: $20.3 \pm 4.8\%$ of enlarged osteocytes in the total osteone, uremia: $19.1 \pm 4.72\%$). Since the ratio of the area of the inner part of the Haversian system to the total area of the Haversian system (neglecting the central canal) is

$$\frac{\frac{r^2}{2} \times \pi}{r^2 \times \pi} = \frac{1}{4}$$

the measured fractions do not differ significantly from the value expected ($= 25\%$). Enlarged osteocytes therefore are randomly distributed in the Haversian systems and do not accumulate in the outer and older parts.

The classification of osteocytes into different categories is a subjective decision. The soundness of the criteria for the decision may be tested by testing the repro-

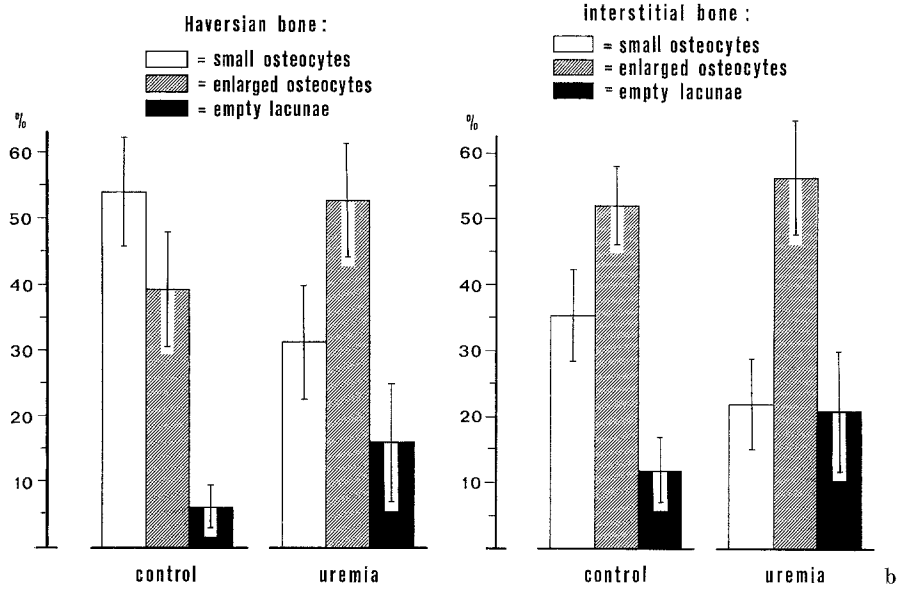

Fig. 2a and b. Histogram showing the percentage of small osteocytes, enlarged osteocytes and empty lacunae in Haversian (a) and interstitial lamellae (b) of human femoral cortical bone

Table 1. Results of osteocyte differential counts in human cortical femoral bone of uremic patients and of patients without skeletal disease

	Control (%)	Uremia (%)	Students t test
Haversian bone			
Small o.	54.0 $\pm$ 8.28	31.2 $\pm$ 8.59	$p<0.001$
Enlarged o.	39.2 $\pm$ 8.48	52.7 $\pm$ 8.70	$p<0.001$
Empty lacunae	6.09 $\pm$ 3.12	15.9 $\pm$ 8.99	$p<0.001$
Interstitial bone			
Small o.	35.6 $\pm$ 7.36	22.1 $\pm$ 9.18	$p<0.001$
Enlarged o.	52.0 $\pm$ 6.05	56.3 $\pm$ 8.75	$p<0.05$
Empty lacunae	11.8 $\pm$ 5.1	21.1 $\pm$ 9.18	$p<0.001$

ducebility of the method. We therefore calculated the 95% limits for individual observations after Bowker (Table 2). 10 repeat differential counts of 100 osteocytes in 7 femura were performed and the limits were calculated by transforming counts to a normal distribution and using factor k 4 (Geigy Table, p. 44). The confidence intervalls for the mean values of osteocytes differential counts in normal bones are given in Table 3.

Table 2. Tolerance limits for osteocyte counts in femoral cortex of normal and uremic patients ([a] mean and 95% tolerance limits after Bowker)

	Patients number	Haversian bone		
		Enlarged o.	Small o.	Empty lacunae
Controls	1	26 (12–44)[a]	64 (46–82)	10 (1–24)
	2	42 (22–70)	52 (40–68)	4 (0–22)
	3	42 (32–50)	50 (42–64)	6 (0–24)
Uremic	1	50 (42–60)	32 (22–42)	18 (16–22)
	2	36 (16–86)	24 (14–32)	40 (22–66)
	3	54 (44–66)	40 (24–60)	6 (0–22)
	4	52 (46–60)	36 (24–42)	12 (4–24)

Table 3. 95% confidence intervall for osteocyte differential counts in normal femoral bone

	Enlarged o.	Small o.	Empty lacunae
Haversian bone	38.5 (36–41)	55.1 (57–63.2)	5.0 (3.6–6.3)
Interstitial bone	66.2 (63.8–68.5)	19.3 (17.2–21.5)	14.8 (12.3–17.4)

Discussion

The above differential counts of osteocytes in femora of healthy individuals are in good agreement with data obtained by Baud and Auil (1971) in human alveolar bone and by Frost (1960) in cortical bone of different location. Our results show that chronic renal insufficiency leads to profound changes in the osteocytic differential count in lamellar cortical bone. The fraction of large activated osteocytes and the fraction of empty lacunae rises at the expense of small osteocytes both in lamellar Haversian and in interstitial bone (Fig. 2a, b; Table 1).

According to Jowsey (1968) it is important to distinguish between enlarged osteocytes in lamellar bone, which are evidence of increased metabolic activity, and large osteocytes, which are found in woven bone, built in disorderly accelerated osteogenesis. Osteocytes in woven bone are former fibrous osteoblasts, which are scattered more or less at random in the matrix. Enlarged cytoplasmic seams and swollen nuclei are part of their normal appearance. In addition they appear to be unresponsive to endocrine and metabolic stimuli (Frost, 1964).

Activation of osteocytes was recognized first by v. Recklinghausen (1910) in rickets and osteomalacia. He coined the term "tryptische Onkose". Later onkosis has been thought to be primarily a necrobiotic phenomenon which anteceeds cell death (Wettstein, 1947). Recent experimental data (Remagen, 1970) suggest that onkosis may reflect necrobiosis of cells exhausted by excessive stimulation as well as reversible activation of osteocytes. The latter contention is supported by the finding of Majno and Roullier (1952) that alkaline phosphatase, which is present in osteoblasts and is lost in neutral osteocytes, reappears in onkotic osteocytes.

When studied by electron microscopy, osteocytes in metaphyseal bone of rat tibiae apparently pass through various stages of maturation, in which they carry out different functions (Jande and Belanger, 1971). According to these authors the life cycle of an osteocyte consists of an early formative period, where the osteocytes resembles osteoblasts. This is followed by a second phase of osteocytic osteolysis, upon which the cell will finally degenerate and die. Whether these findings in metaphyseal bone of rats are representative for bone in general, is a moot question. In cancellous bone of human iliac crest for instance one sees closely interspersed active osteocytes as well as empty lacunae or neutral osteocytes without any layering (Vittali, 1968).

This finding would argue against a preprogrammed irreversible sequence of functional states.

Activated osteocytes are able either to destroy mineralized bone matrix by the process of osteolysis or to synthesize new bone matrix (Belanger, 1965; Baylink and Wergedal, 1971). The preeminent importance of osteocytic activity for the regulation of serum calcium levels seems to be firmly established today (Neuman and Ramp, 1971). Simple arythmetics show that the release of calcium by osteoclastic resorption represents only a minute fraction of total calcium efflux from the skeleton, the difference being accounted for by calcium transport across unspecified cellular membranes. The latter presumably also include osteocytes. In uremia there is a peculiar discrepancy between the histological evidence of increased activation of osteocytes with increased osteocytic osteolysis on one hand and the inability of the uremic skeleton to maintain normal serum calcium levels (hypocalcemic hyperparathyroidism, Stanbury, 1968) on the other hand. The paradoxial inability of activated osteocytes to remove adequate amounts of calcium from the skeleton might point to impaired cellular efficiency similar to what has been found for osteoclasts in clinical (Jaworski *et al.*, 1969; Villanueva *et al.*, 1970) and experimental uremia (Krempien *et al.*, 1972a). The histological finding of an increased number of activated cells certainly does not give information about the rate of osteolysis.

Some of the alternative explanations seem less likely. Since the total amount of unmineralized bone matrix has been shown to be increased in chronic renal insufficiency (Garner and Ball, 1966), bone mineral released by activated osteocytes might well be trapped in newly formed osteoid (internal shift). Finally since azotemic bone is undermineralized as shown by micro-radiography (Jowsey, 1969) and by measurements of microhardness (Krempien *et al.*, 1972c), it is conceivable that the amount of bone mineral in the bone volume under the influence of a given activated osteocyte is reduced. It should also be pointed out that the resistance of the skeleton to the calcemic action of parathyroid hormone (Evanson, 1966), which is commonly found in renal failure, must involve osteocyte malfunction.

In renal insufficiency serum parathyroid hormone levels are markedly elevated (Reiss *et al.*, 1969). Parathyroid hormone is known to activate osteocytes and to enlarge osteocytic lacunae by periosteocytic osteolysis (Belanger, 1965). Meunier *et al.* (1971) found marked widening of osteocytic lacunae in hyperparathyroidism. In good agreement with this finding a reasonable correlation was observed between the size of osteocytic lacunae and the height of serum para-

thyroid hormone levels in cancellous bone of patients with renal insufficiency, who were examined in one of our previous studies (Krempien *et al.*, 1972b).

The accumulation of empty lacunae as evidence of osteocytic death suggests that in renal insufficiency the survival time of osteocytes is shortened presumably under the influence of excessive parathyroid hormone activity. In an electron microscopic study Jande (1972) observed an increase of the fraction of resorptive and degenerating osteocytes associated with a decrease of the fraction of formative osteocytes under the influence of parathyroid hormone. An shortened survival time of osteocytes as a consequence of excessive stimulation should lead to an increase of the fractions of activated and dead osteocytes at the expense of the fraction of neutral osteocytes. This is exactly the change of osteocyte pattern that was found in the present investigation. Empty osteocytic lacunae as evidence of osteocytic death are randomly distributed over the cross sectional area of an Haversian system and do not accumulate in the peripheral and older parts. This finding would suggest that osteocyte death is a random event.

In contrast to what is seen in Haversian bone, the increase of activated osteocytes was less pronounced in interstitial extra-Haversian bone of azotemic patients. This finding may simply reflect the fact that the pool of neutral precursor osteocytes in interstitial bone, i.e. the remnants of former Haversian systems has already been exhausted previously. It is therefore not surprising to find increased accumulation of dead osteocytes under the influence of parathyroid hormone in this site. If the presence of dead osteocytes triggers osteoclasia, as suggested by Frost (1962), one might speculate whether the acceleration of osteocytic life cycle with the consequent accumulation of dead osteocytes might not be the event triggering increased cortical bone remodelling in uremia.

In conclusion, activation of osteocytes is one of the most sensitive indicators of parathyroid hormone activity in the skeleton. It is therefore also of diagnostic value. In addition, osteocyte malfunction must be the major determinant of the faulty regulation of serum calcium levels in chronic renal insufficiency. This is particularly remarkable, since osteocytes appear to be activated histologically in spite of their disturbed capacity to liberate mineral from the skeleton. Since hypocalcemia induces hyperparathyroidism, a vicious circle is established, that leads to progressive destruction of the premorbid skeleton by hyperparathyroid bone disease.

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