Effects of Iron Overload on Bone Remodeling in Pigs

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For study of the effects of an iron overload on bone remodeling, 5 control pigs were compared with 5 pigs given a total dose of 10.8 g of parenteral iron in 36 days. Treated pigs developed an iron tissue overload demonstrated by a marked increase in bone and liver iron. Except for a modest increase in SGOT, there was no biochemical or histologic sign of liver damage. Serum levels of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D were unchanged in the treated pigs. There was no accumulation of iron in the parathyroid glands and the serum immunoreactive parathyroid hormone level was unchanged in the treated animals. Bone histomorphometry after double tetracycline labeling showed that in the treated pigs osteoblast cell surfaces, double and total labeled surfaces, appositional rate, and formation at tissue level were significantly decreased, and reversal surfaces were increased. Mineralization was not impaired because the osteoid thickness was unchanged. From the morphometric measurements it was concluded that osteoblast recruitment and the collagen synthesis rate were decreased. Mean wall thickness, which indicates the amount of bone synthesized, was also lowered. In contrast, the osteoclastic resorption surfaces and the depth of lacunae resulting from osteoclast resorption were unchanged by treatment. Despite this imbalance between formation and resorption, trabecular bone mass estimated on trabecular bone volume and bone ash was unchanged after 36 days' treatment. Perls' stain revealed that iron deposits were present in osteoblast and osteoclast cells and also inside the bone matrix, because there was a linear deposit along the trabecular surfaces, cement line, and osteoid–mineralized bone interface. Therefore, because treatment induced no modification of the major humoral regulators of bone metabolism, it is suggested that iron, which was present in bone cells and matrix, could play a role in bone remodeling. (Am J Pathol 1984, 116: 377–384)

ALTHOUGH an iron overload in human pathology may be associated with osteoporosis, it cannot be inferred that iron plays a role in bone changes. Osteoporosis in primary hemochromatosis may be related to multiendocrine deficiency[1,2] and in Bantu hemochromatosis to vitamin C deficiency.[3] Furthermore, in both cases, hepatic failure, frequently associated with bone loss, may also induce osteoporosis.[4] In thalassemia major with secondary hemochromatosis, impaired bone mineralization and apposition associated with iron bone overload have been observed.[5] The role of iron in such disorders, however, has not been demonstrated. At the present time, an iron overload is also frequently observed in hemodialyzed patients,[6] and it is relevant to know whether an iron overload may play a role in renal osteodystrophy.

We therefore induced an iron overload in pigs of the same magnitude as that observed during human hemochromatosis. Bone remodeling after 36 days of iron overload was studied in growing pigs after double tetracycline labeling[7] for study of whether this treatment triggers bone changes.

Materials and Methods

Experimental Procedure

Ten female LW pigs aged from 76 to 91 days, weighing 33.7 ± 0.8 kg, fed a classic diet (0.8% Ca, 0.6% P, and 2000 IU of vitamin D_3 per kilogram diet), were divided into two groups. Five pigs were untreated, and 5 pigs received 300 mg of dextran iron per day intramuscularly for 36 days (ie, a total of 10.8 g per pig). All the animals were killed at the end of the experiment (day 36).

Biochemical Methods

Blood samples were collected three times: at the beginning of the experiment (day 0), 3 weeks later

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(day 21), and at slaughter (day 36) for study of the serum kinetics of iron, calcium, phosphate, magnesium, and alkaline phosphatases, bilirubin, serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), γ-glutamyl transferase (γ-GT), siderophilin saturation, and 25-hydroxyvitamin D (25-OHD) serum levels were determined in slaughter samples by methods described elsewhere.[5,8] At slaughter, serum 1,25-dihydroxyvitamin D (1,25-$[OH]_2D$) was determined according to the method of Sheppard and De Luca,[9] serum vitamin C was determined according to the method of Roe and Khuether,[10] and plasma parathyroid hormone (PTH) was measured by radioimmunoassay with PTH RIA 200 kits (courtesy of CEA, Saclay, France) with a bovine system; the antibody was specific against the PTH carboxyl terminal. Liver iron was determined by Pr. Brissot (Hôpital Ponchaillou, Renne, France).[11,12] Two epiphyses of main metatarsal bones were used for evaluation of bone mineral content (ash, calcium, phosphorus, and iron). Two-day urine samples were collected during the last week experiment for calcium, phosphorus, and hydroxyproline determination.[5]

Table 1—Biochemical Parameters of Iron Overload and Liver Function

	Control pigs	Treated pigs
Plasma iron (μg/dl)		
Day 0	170.8 ± 31.6[a]	132.7 ± 8.6[a]
Day 21	136.1 ± 6.5[a]	274.4 ± 35.1[b]
Day 36	149.0 ± 9.0[a]	368.5 ± 46.5[c]
Siderophylin saturation (%)	23.9 ± 1.5	37.8 ± 1.8*
Liver weight (g)	1102 ± 40	1270 ± 37*
Liver iron μg/100 mg dry weight	24 ± 6	586 ± 91†
SGOT (UI/l)	33.6 ± 3.4	53.8 ± 6.4*
SGPT (UI/l)	37.4 ± 6.3	42.2 ± 8.2
Serum bilirubin (μg/dl)	164 ± 29	117 ± 16
Serum γ GT (UI/dl)	3.74 ± 0.63	4.22 ± 0.82

All results are expressed as mean ± SEM. Plasma iron values with common superscript letters are not different according to the Student range Q test at the 5% level.
* $P < 0.02$, variance analysis.
† $P < 0.01$.

Histologic Methods

The two parathyroid glands were weighed. One gland was frozen for lipid staining, and the other was routinely fixed and embedded. The liver was weighed, a fragment was preserved for iron determinations, and the remainder was routinely fixed and embedded.

The pigs were subjected to double tetracycline labeling with 20 mg/kg of body weight oxytetracycline intramuscularly for each labeling procedure, according to the following schedule: 2 days on, 7 days off, and 1 day on. The pigs were slaughtered 2 days after the last labeling procedure. A fragment of trabecular bone from the iliac crest was removed, kept undecalcified, embedded in methyl methacrylate, and sectioned with a Jung K microtome. Five-micron-thick sections were cut and stained with toluidine blue, and 15-μ-thick sections were kept unstained for tetracycline fluorescence evaluation. We measured the trabecular bone volume in an area of 15 sq mm on three sections, 2 mm below the cortex, at a site located just anterior to the anterosuperior iliac crest. The percentage of trabecular surfaces covered with osteoid seam was measured at a magnification of ×350. We measured osteoblast surfaces, taking into account plump osteoblasts, and expressed the results as the percentage of total trabecular surfaces. We measured osteoclast resorption surfaces, taking into account the trabecular surfaces covered by osteoclasts, and expressed the results as the percentage of total trabecular surfaces. Howship lacunae devoid of osteoclasts were expressed as the percentage of total trabecular bone surfaces and denominated reversal surfaces.[13]

The depth of the lacunae resulting from osteoclast resorption was measured with an image analyzer. The line of an integrating eyepiece was positioned parallel to the two edges of the resorption lacuna, and we measured the distance from the deeper point of the resorption cavity to the line of the eye gird. Mean osteoid thickness was measured at a magnification of ×500 with an image analyzer at equidistant points, which were selected by the intersection of the parallel lines of an integrating eyepiece with the trabecular surfaces. Mean wall thickness is the thickness of a complete packet[14] and was measured in the same way as mean osteoid thickness at equidistant points. We measured, on 15-μ undecalcified sections, single and double labeled trabecular surfaces and expressed the results as the percentage of total trabecular surfaces. Total labeled surfaces were determined according to the formula

$$\text{Double labeled} + \frac{\text{Single labeled surfaces}}{2}.$$

The appositional rate was measured also at an equidistant point. From these parameters we calculated formation at the tissue level using the formula

$$\text{Total labeled surfaces} \times \text{appositional rate}$$

and the duration of the formation period (sigma F) using the formula[7]

Table 2—Evolution of Serum Calcium, Phosphate, Magnesium, and Alkaline Phosphatase During the Experimental Study

	Day 0	Day 21	Day 36
Serum calcium (mg/dl)			
Treated	10.7 ± 0.2^{ab}	10.2 ± 0.3^{b}	9.8 ± 0.1^{b}
Control	11.4 ± 0.1^{a}	11.2 ± 0.4^{a}	10.2 ± 0.2^{b}
Serum phosphate (mg/dl)			
Treated	8.5 ± 0.2^{ab}	7.8 ± 0.4^{b}	7.6 ± 0.4^{b}
Control	9.1 ± 0.5^{a}	8.1 ± 0.2^{ab}	7.0 ± 0.3^{b}
Serum magnesium (mg/dl)			
Treated	1.8 ± 0.03^{a}	1.5 ± 0.15^{a}	1.7 ± 0.05^{a}
Control	1.7 ± 0.04^{a}	1.6 ± 0.03^{a}	1.7 ± 0.02^{a}
Alkaline phosphatases (UI/l)			
Treated	251 ± 33^{a}	184 ± 17^{a}	251 ± 33^{a}
Control	234 ± 17^{a}	267 ± 33^{a}	251 ± 33^{a}

All results are expressed as the mean ± SEM. Values with common superscript letters are not different according to the Student range Q test at the 5% level.

$$\frac{\text{Mean wall thickness}}{\text{Appositional rate}}.$$

The parathyroid, hepatic, and bone sections were colored with Perls' stain for iron detection.[15]

Statistical Methods

The Student Range Q test (Neuman-Keuls) was used for multiple mean comparisons (plasma kinetics).[16] At slaughter the two groups' results were compared by variance analysis. All results are expressed as the mean ± SEM.

Results

Iron treatment did increase the iron content of the liver significantly. There was no treatment effect on the growth rates (slaughter weight: 58.2 ± 1.8 control versus 56.5 ± 1.5 kg treated, $P < 0.05$). As indicated in Table 1, there was a linear and dramatic increase of the serum iron levels in treated pigs. Iron treatment did increase serum siderophilin saturation and iron content of the liver. Table 1 shows also that only the SGOT level was elevated by iron overload; whereas the SGPT, bilirubin, and γ-GT serum levels remained unchanged. The liver weight was increased in treated animals.

Hepatic sections did not show any fibrosis or inflammatory processes. Perls' stain showed iron deposits occurring primarily in the centrolobular region. Large irregular iron deposits were observed in the Kupffer cells and small regular blue granules in the hepatocytes, which at times had a diffuse pale blue coloration. None of the liver sections from the control pigs stained blue with Perls' stain.

Table 2 shows that during the study serum calcium and phosphate levels decreased in the control pigs, which did not happen significantly in iron-treated pigs. However, with the exception of serum calcium on Day 21, which was higher in the controls than in treated pigs, there was no significant difference between the two groups for plasma calcium, phosphate, magnesium, and alkaline phosphatases values, whatever the period.

Table 3 shows that 24-hour urine calcium, phosphate, and hydroxyproline were not affected by the iron treatment. The serum 25-OHD levels (6.8 ± 2.5, control, versus 6.8 ± 1.5 ng/ml, treated) and serum 1,25-$(OH)_2$D levels (68.7 ± 7.4, control, versus 61.5 ± 2.8 pg/ml, treated) were not different between the two groups. Serum iPTH levels (1.6 ± 0.5, control, versus 2.0 ± 0.5 ng/ml, treated) were not modified by treatment. Serum vitamin C (0.72 ± 0.22, control, versus 0.66 ± 0.18 mg/l, treated) was low, but unchanged in the two groups.

The average parathyroid weights were not significantly different between the two groups (62 ± 9, control, versus 64 ± 14 mg, treated). Staining of frozen sections with oil red O revealed the absence of hyperplasia in both groups. No iron deposit was detectable in treated pigs with Perls' stain. Bone calcium, phosphate, magnesium, and ash content was unchanged. Bone iron content was markedly increased in treated pigs (Table 4), and there was a significant correlation between liver and bone iron ($r = 0.89$, $P < 0.05$, n = 5).

Bone histomorphometry after double tetracycline labeling (Table 5) showed that trabecular bone volume was unchanged in treated pigs. Osteoid surfaces were decreased in treated pigs. The osteoid thickness remained the same in both groups. The osteoblast, double, and total labeled surfaces dwindled in treated pigs. The appositional rate was slightly reduced in treated animals. As a result of the lower appositional rate of each osteoblast and the decrease in

Table 3—Twenty-four-hour Urine Calcium Phosphate and Hydroxyproline at the End of the Study (Day 36)

	Control pigs	Treated pigs
24-hour urine calcium (mg)	152 ± 22	166 ± 24
24-hour urine phosphate (mg)	313 ± 75	247 ± 54
24-hour hydroxyproline (mg)	198 ± 25	172 ± 16

Results are expressed as the mean ± SEM. There was no significant difference between treated and control pigs.

Table 4 – Bone Mineral Content of Metatarsal Bones

	Control pigs	Treated pigs
DM, %WW	69.68 ± 1.08	68.13 ± 1.82
Ash (% DM)	48.26 ± 0.86	47.89 ± 0.51
Calcium (% DM)	17.18 ± 0.26	16.98 ± 0.47
Phosphate (%DM)	8.59 ± 0.11	8.32 ± 0.19
Magnesium (%DM)	0.31 ± 0.11	0.31 ± 0.01
Iron (PPM of DM)	34.10 ± 1.18	360.40 ± 42.8*

Results are expressed as the mean ± SEM. WW, wet weight. DM, dry matter.
* Significant difference between treated and control pigs ($P < 0.01$).

Table 5 – Bone Histomorphometry

	Control pigs	Treated pigs
Trabecular bone volume	18.1 ± 1.7	17.4 ± 2.0
Osteoid surfaces (%)	31.1 ± 3.7	19.7 ± 3.2*
Osteoblast surfaces (%)	19.0 ± 1.8	8.5 ± 2.3†
Mean osteoid thickness (μm)	4.8 ± 0.4	4.8 ± 0.2
Double labeled surfaces (%)	37.9 ± 1.2	30.9 ± 3.1*
Single labeled surfaces	11.4 ± 1.1	13.9 ± 1.7
Total labeled surfaces (%)	42.8 ± 1.9	36.4 ± 2.0†
Appositional rate (μm/day)	2.6 ± 0.14	2.1 ± 0.10†
Formation at tissue level (μ^3/μ^2/day)	1.121 ± 0.09	0.808 ± 0.07*
Mean wall thickness (μm)	40.9 ± 2.1	33.2 ± 2.4†
Sigma F day	15.7 ± 0.3	15.4 ± 0.2
Reversal surfaces (%)	2.8 ± 0.5	4.7 ± 0.7*
Osteoclast resorption surfaces (%)	5.5 ± 0.9	4.6 ± 0.72
Depth of resorption lacunae (μm)	8.0 ± 0.4	8.3 ± 0.3

Results are expressed as the mean ± SEM.
* $P < 0.01$.
† $P < 0.05$. (significant difference between treated and control pigs).

active forming surfaces, the formation rate at tissue level was markedly reduced in treated pigs. The mean wall thickness reflecting the quantity of bone deposited during one remodeling period was decreased in treated pigs. The duration of the formation period was 15.7 ± 0.3 days in control and 15.4 ± 0.2 days in treated pigs and thus not significantly different in the two groups. Reversal surfaces were significantly increased by iron overload. Unlike the results concerning bone formation, no substantial difference was noticeable for bone resorption in the treated pigs. The osteoclast surfaces and the depth of lacunae resulting from resorption were unchanged in the two groups.

On sections from the five control pigs stained with Perls' stain, there was no sign of a blue deposit, whereas on sections from the five treated pigs, there was a linear deposit of iron inside the bone, at the edge of the trabecular surfaces and at the osteoid/mineralized bone interface (Figure 1). Also, an iron blue deposit could seldom be detected at the cement line. Iron was present as a large irregular deposit in marrow macrophages. Histochemically stained iron could also be detected in bone cells. In the osteoclasts, there were infrequently large irregular deposits, similar to those seen in marrow macrophages (Figure 2); but most of the time, the osteoclasts were found to have a diffuse pale blue color (Figure 3). The osteoblasts contained small blue granules (Figure 4).

Discussion

The iron overload produced in our study by acute means has avoided rough liver damage, which could have influenced the bone metabolism. In a previous

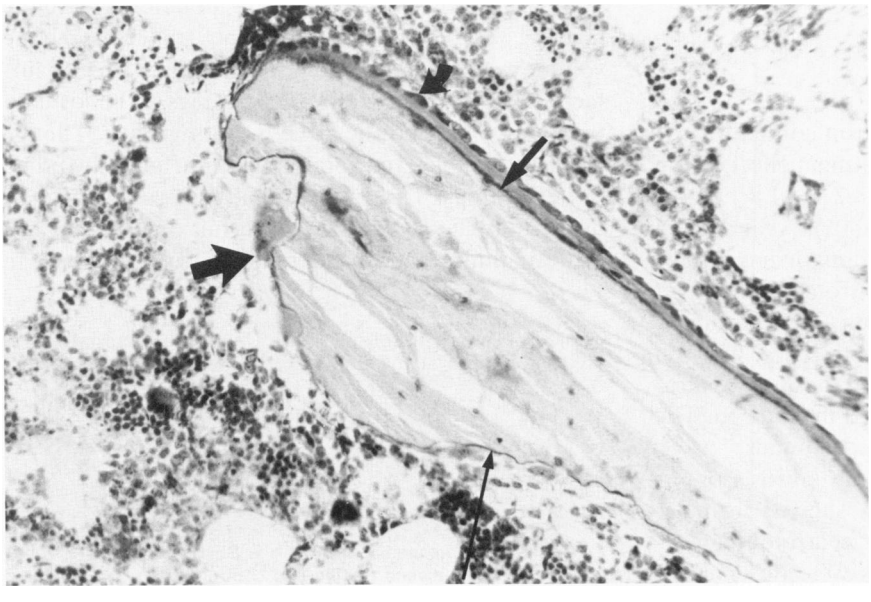

Figure 1 – ⟶, Iron deposit at the edge of the trabecular surface. ⟶, Iron deposit at the interface of osteoid and mineralized bone. ↘, osteoblast. ➡, osteoclast with large iron deposits. (Perls' stain, counterstained with May-Grünwald-Giemsa, ×50)

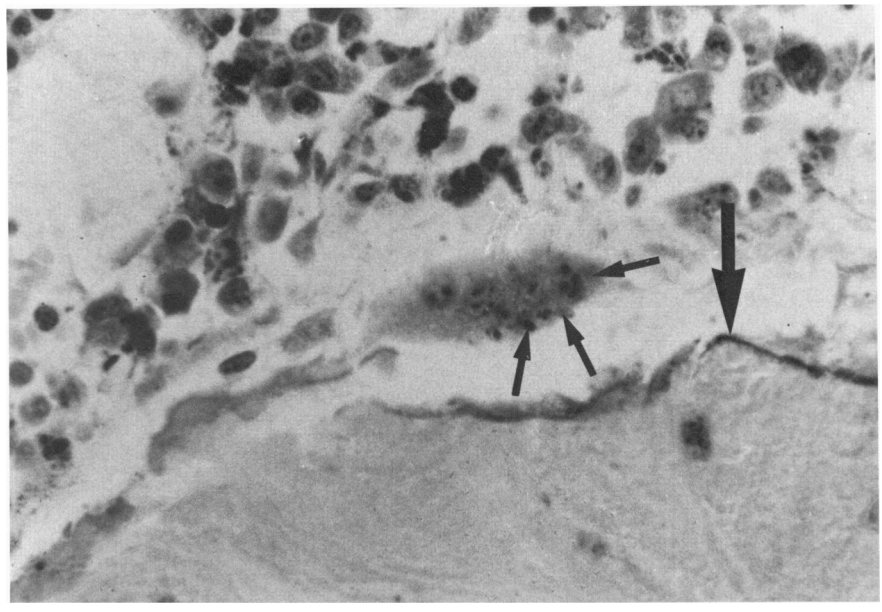

Figure 2 —→, Big irregular iron deposit inside osteoclast. ⟶, linear deposit at the edge of the trabecula. (Perls' stain, counterstained with May-Grünwald-Giemsa, ×400)

experiment, Lisboa[17] induced cirrhosis in dogs by parenteral injections of iron, using the same total dose range as in our pigs; but in his case, the iron overload resulted from a mean 12 months' treatment instead of the 36 days' treatment in our study. Similarly, in HLA-related hemochromatosis[18] and transfusional iron overloading,[19] many years elapse before hepatic lesions develop. In fact, in our experimental study, we obtained a massive iron overload because liver iron concentrations in the pigs were within the same range as those found in adult men with primary hemochromatosis.[12] Only slight liver parenchymal damage was observed, if one considers the very slightly elevated SGOT, the unchanged SGPT γ-GT serum levels, and the absence of histologic alteration. No modification of bone metabolism has been reported in such cases. We obtained iron concentrations of the same magnitude both in macrophages and parenchymal cells of the marrow and liver. This type of iron distribution is similar to that observed in massive secondary hemochromatosis, because of the identical marrow and liver overload and of the localization of iron in macrophage cells,[20,21] but there was also a parenchymal iron overload similar to the one observed during HLA-related hemochromatosis.[12]

In controls, serum calcium and phosphate levels

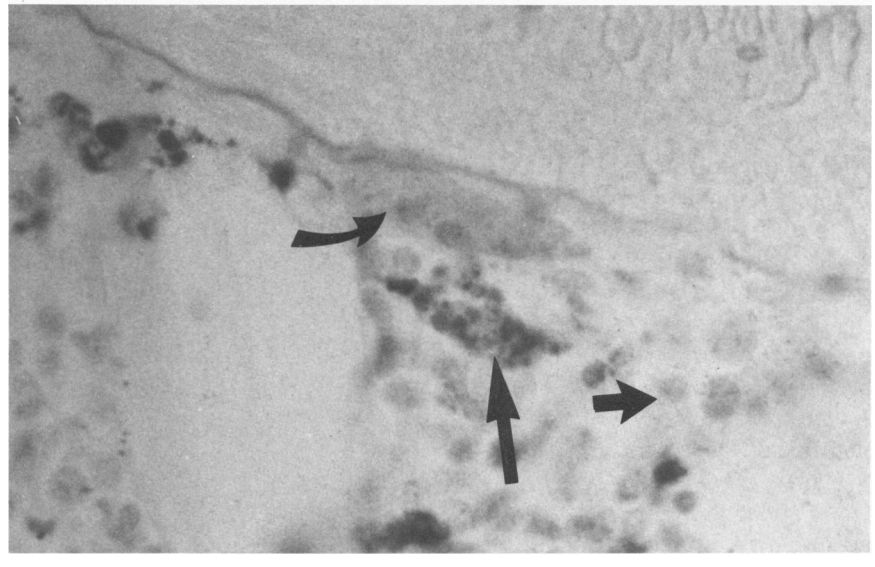

Figure 3 — ⟶, diffuse pale blue color of an osteoclast cytoplasm. ⟶, big irregular deposits in marrow macrophages. ➡, marrow cells nucleus with uncolored cytoplasm. (Perls' stain, counterstained with solid red, ×400)

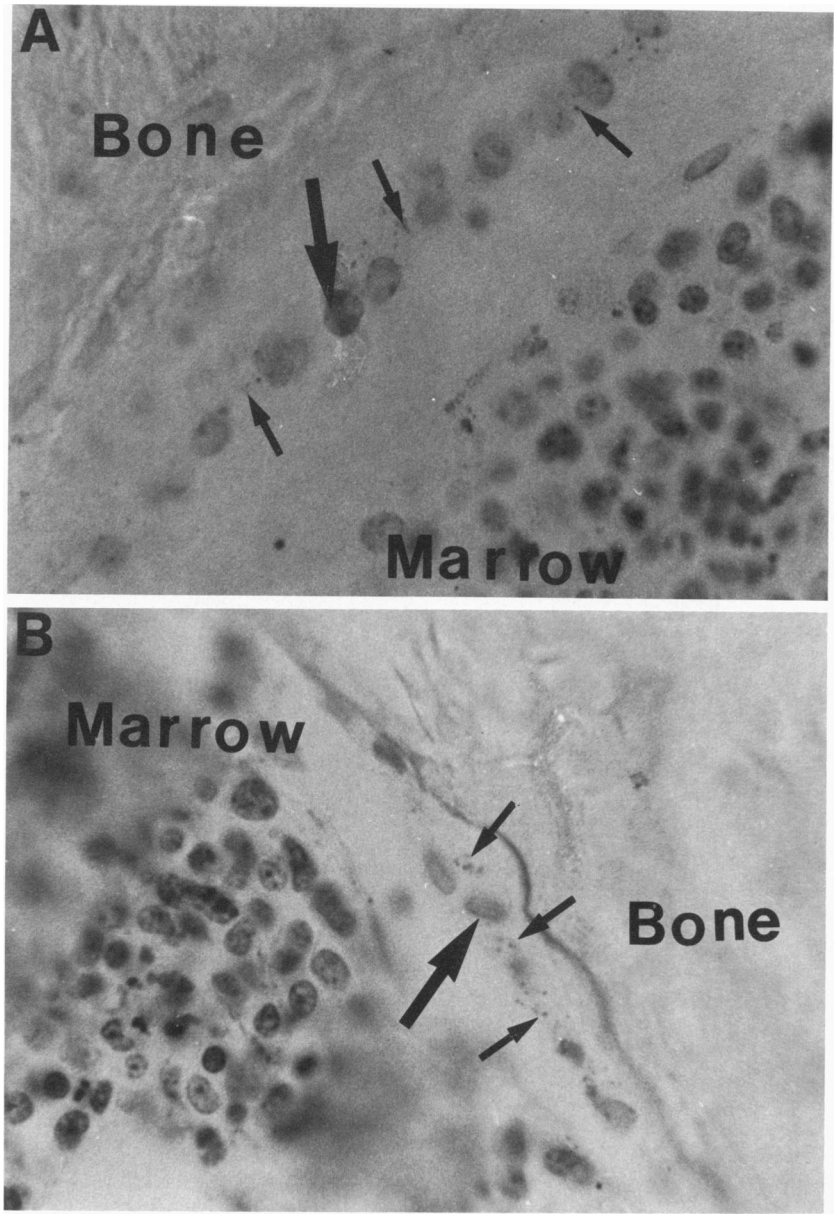

Figure 4 — ➡, osteoblast nucleus. ←, small blue deposits inside the cells. Osteoid is uncolored. (Perls' stain, counterstained with safranin picric alcohol, ×400)

decreased significantly during the study, as usually observed in growing pigs.[22] There is no clear explanation in the whole results for the lack of significant decrease between Day 21 and Day 36 in treated, as compared with control, pigs. From the beginning of the study serum calcium was higher in control than in treated pigs, despite the random distribution of the 10 pigs into two groups. This difference in serum calcium between the two groups reached significance at Day 21, and no special meaning can be given to this transient result.

Iron overload did not induce any change in 25-OHD and $1,25(OH)_2D$ serum levels. The parathyroid hormone secretion, estimated by anatomic and biochemical methods, was unchanged in the treated pigs; whereas it has been reported in human hemochromatosis to be increased secondary to low vitamin D levels[23] or decreased because of iron deposits in the parathyroid tissue.[24] Thus, the histologic changes we observed in bone remodeling cannot be interpreted as resulting from modification of the two major regulators of calcium phosphate metabolism, ie, vitamin D and parathyroid hormone. Furthermore, the vitamin C serum levels were unchanged by treatment.

On the basis of biochemical parameters (alkaline phosphatases and hydroxyprolinuria) or morphometric parameters, bone remodeling was about four times higher in our animals than in adult men. There

was no evidence of mineralization impairment in the treated pigs. Although the appositional rate, also called the mineralization rate,[25] was decreased, osteoid thickness remained unchanged. In such cases a mineralization impairment cannot be demonstrated, since it is difficult to believe that the osteoid lamellae deposited slowly by the osteoblast at one edge of the osteoid seam can be mineralized at a higher rate at the interface of osteoid and mineralized bone. Both a decrease in a dynamic parameter if one takes the appositional rate into account, and an increase in osteoid thickness are required to demonstrate mineralization impairment[25]; this did not happen in the pigs treated by iron.

The most impressive changes in bone remodeling concerned formation. In treated pigs, the osteoblast and double labeled surfaces were markedly lower. This decrease in osteoblast number might be related to decrease either in osteoblast recruitment or in osteoblast life span. The length of the formation period (sigma F) reflects the life span of a group of osteoblasts at one remodeling cycle.[26] Thus, because sigma F was unchanged in treated pigs, the life span of osteoblasts was unchanged by treatment. On the other hand, reversal surfaces which were of primary importance in the local coupling between resorption and formation grew substantially.[13] This growth in reversal surfaces already observed during osteoporosis may be interpreted as a delay in the onset of osteoblast formation.[27] Thus, a decrease in active formation surfaces depended on decreased osteoblast recruitment.

The second most important aspect of the formation results was lowered mean wall thickness, which reflects decreased quantity of bone deposited during one remodeling cycle. This was related to a decrease in both the extent of active formation surfaces and the appositional rate. No significant decrease could be observed either in osteoclastic resorption surfaces or in osteoclast activity according to osteoclastic resorption depth. This imbalance between maintained resorption and decreased quantity of deposited bone should have induced a decreased bone mass[28]; however, we did not observe any modification of trabecular bone volume or of bone ash content. This discrepancy can be interpreted in two ways. The first hypothesis is that we were not able to detect an effective decrease in resorption (osteoclastic resorption surfaces and hydroxyprolinuria were slightly, but not significantly, decreased; and osteoclastic resorption depth does not reflect entirely the resorption rate). The second hypothesis, which seems more likely, is that the length of the experience was too short to give us the opportunity to detect a decrease in trabecular bone volume or bone ash content, which are not very sensible methods of measuring trabecular bone mass.

The histochemical demonstration of iron by Perls' stain is a reliable method.[15] The iron deposit observed in treated pigs inside the trabecular bone and cement line have already been reported in children with thalassemia.[15] This experimental study demonstrated that the iron deposit at the mineralized bone–osteoid interface did not block mineralization, because there was no mineralization impairment in the treated pigs. In human hemochromatosis iron deposits have only been demonstrated in osteoclasts.[5] The massive acute overload achieved in this experimental study led to a stainable iron deposit in both kinds (osteoblasts and osteoclasts) of bone cells. Some iron deposits in osteoclasts were large and irregular, usually considered to be hemosiderin,[15] similar to those observed in marrow macrophages. This bears out the hypothesis that marrow macrophages might be local osteoclast precursors.[29] Usually, however, the osteoclasts had a diffuse pale blue color, and osteoblasts contained small regular granules. Both kinds of histochemical deposits are typical of primary hemochromatosis[15] and, according to Richter and Bessis,[30] indicate a ferritin deposit. The presence of iron in the osteoclasts did not seem to modify osteoclast activity. On the other hand, iron inside the osteoblast might play a role in the lowered osteoblast activity observed. Local mechanism for osteoblast recruitment is at the present time poorly understood. However, iron might also play a role in decreased osteoblast recruitment, either by accumulation in bone matrix at cement line or by iron accumulation in the macrophage lineage cells, which exists at the reversal surfaces.[13]

Finally, a massive iron overload leads to decreased osteoblast numbers and activity. This could be related to the presence of iron inside the bone cells and along the trabecular bone surfaces. It is not known whether the role iron plays in remodeling is of primary importance in human pathology when iron overload occurs more progressively.

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