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The role of bone biopsy in clinical practice and research

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The role of bone biopsy in clinical practice and research. Survival rates of patients on dialysis have increased with improved dialytic therapy. However, the resultant increased duration of dialysis has led to a rise in renal osteodystrophy (ROD). Because this metabolic bone disease can produce fractures, bone pain, and deformities late in the course of the disease, prevention and early treatment are essential. Types of ROD include predominant hyperparathyroid bone disease, low turnover bone disease (including osteomalacia and adynamic bone disease), and mixed uremic osteodystrophy. Serum PTH levels are commonly used to assess bone turnover in dialyzed patients. However, a recent study in our laboratory found that serum PTH levels between 65 and 450 pg/ml seen in the majority of dialysis patients are not predictive of the underlying bone disease. To date, bone biopsy is the most powerful and informative diagnostic tool to provide important information on precisely the type of renal osteodystrophy affecting patients, the degree of severity of the lesions, and the presence and amount of aluminum deposition in bone. Bone biopsy is not only useful in clinical settings but also in research to assess the effects of new therapies on bone. The methods of *in situ* hybridization histochemistry (ISHH) and immunohistochemistry (IHC) are providing the means to study local biomolecules that play a role in bone metabolism. As these research tools become more refined, they will become increasingly valuable in the study of bone. Alternatives to the bone biopsy continue to be pursued, but they have not been proven to have the same specificity or sensitivity to effectively determine the potential value of a specific therapeutic regimen.

Renal failure produces changes in mineral metabolism that affect bone structure, turnover, and cellular characteristics. When patients reach end-stage renal failure and require chronic maintenance dialysis, nearly all of them have abnormal bone histology. Improved dialytic therapy has increased survival rates of patients on dialysis, but the resultant increased duration of dialysis has led to a rise in renal osteodystrophy, a metabolic bone disease that can produce fractures, bone pain, and deformities late in the course of the disease. Therefore, prevention of renal osteodystrophy is critical. To date, bone biopsy is the most informative diagnostic tool and provides information on the type of renal osteodystrophy, the degree of severity of the lesions, and the presence and amount of aluminum deposition in bone [1].

Bone biopsies have been used for research purposes to assess the effects of new therapies on bone. More recently, the methods of *in situ* hybridization histochemistry and immunohistochemistry are providing the means to study local biomolecules that play a role in bone metabolism.

This article discusses the types and evolution of renal osteodystrophy, presents an overview of bone biopsy techniques, discusses clinical applications of bone biopsy, and reviews the research implications of *in situ* hybridization histochemistry and immunohistochemistry.

TYPES OF RENAL OSTEODYSTROPHY

Despite a mostly common initial pathogenetic pathway, renal osteodystrophy is not a uniform bone disease. The forms of renal osteodystrophy consist of predominant hyperparathyroid bone disease, low turnover bone disease, including osteomalacia and adynamic bone disease, and mixed uremic osteodystrophy. Transformation from one form to another can occur [2].

Predominant hyperparathyroid bone disease

Predominant hyperparathyroid bone disease (PHBD) is characterized by a marked increase in bone turnover. Irregularly shaped trabecules display numerous abnormal remodeling sites (Fig. 1), and an unusually high number of bone cells are irregular in arrangement and shape. Deep, irregular resorption cavities often dissect or tunnel the trabecules. Numerous enlarged osteoclasts contain multiple nuclei with prominent nucleoli. Osteoblast shape changes from cuboidal to polygonal or spindle shaped. The usual palisade-like monolayer of osteoblasts can be replaced by atypical multilayered arrangements of cells with variable orientation toward the bone surface (from parallel to perpendicular). Osteoid surface and volume increase, and an osteoid seam can thicken because of collagen overproduction. The resulting osteoid is primarily of the woven, irregular type. In advanced cases, fibro-

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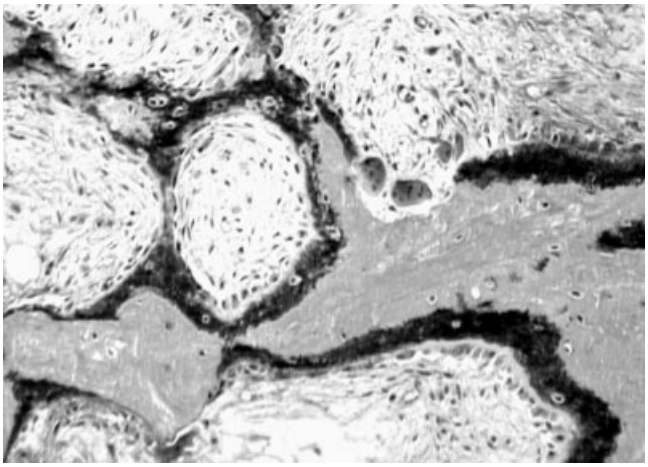

Fig. 1. Predominant hyperparathyroid bone disease. High fraction of trabecular surface covered by osteoid seams. High osteoid–osteoblast interface. High bone–osteoclast interface with appearance of deep resorption lacuna. Marrow fibrosis. Undecalcified, 3 μm thick section of human iliac bone (modified Masson-Goldner stain, ×125 magnification).

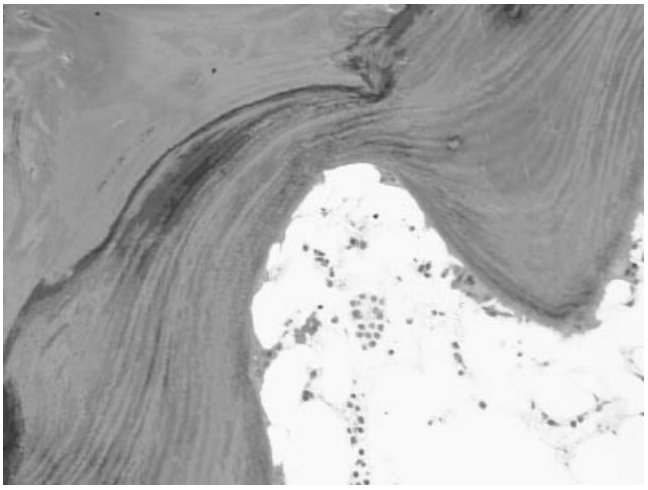

Fig. 2. Low-turnover osteomalacia. Increase in osteoid volume. Wide osteoid seams. Absence of osteoblasts and resorption surface. Undecalcified, 3 μm thick section of human iliac bone (modified Masson-Goldner stain, ×200 magnification).

sis replaces bone marrow entirely, reflecting the irregular activity of osteoblasts in which the regular activity of individual cells is decreased. Numerous irregular osteocytic lacunae within woven osteoid and mineralized bone result from the increased number of osteoblasts entrapped in bone, and the mineral apposition rate and the extent and number of mineralizing sites are notably increased. Findings of thick osteoid seams and diffuse, irregular tetracycline uptake in woven bone are sometimes mistakenly diagnosed as osteomalacia.

A marked increase in bone turnover often leads to cancellization of the cortical bone. This causes a net decrease in cortical bone volume most evident in the appendicular skeleton. PHBD usually presents with high bone volume, but malnutrition, immobilization, or other causes can markedly reduce bone volume. Also, areas of high bone mass may be adjacent to pseudocysts consisting primarily of hyperplastic or fibrotic bone marrow. In any case, bone strength cannot be equated with high bone volume in PHBD patients because the irregular trabeculae may lose their proper three-dimensional architecture and connectivity. Also, the poorly mineralized trabeculae consist mainly of mechanically deficient woven bone with a propensity to fracture. This disease creates a particularly fragile, fracture-prone bone; thus, the use of the term "osteosclerosis" is inappropriate.

Low turnover bone disease

Representing the other end of the spectrum from PHBD, low turnover uremic osteodystrophy is marked by a profound decrease in active remodeling sites. Certain features reflect a marked decrease in osteoblastic activity and there are few osteoclasts and osteoblasts. Usually, only a few thin single labels of tetracycline are observed. Other characteristics include predominantly lamellar bone structure, few active bone formation sites, and markedly reduced mineralizing surfaces.

This type has two subgroups: osteomalacia (LTOM) and adynamic renal bone disease (ABD). LTOM is characterized by an accumulation of unmineralized matrix in which a diminution in mineralization precedes or is more pronounced than the inhibition of collagen deposition (Fig. 2). In LTOM, unmineralized bone makes up a sizable portion of trabecular bone volume. The increased lamellar osteoid volume results from the presence of wide osteoid seams that cover much of the trabecular surface. The occasional presence of woven bone buried within the trabeculae indicates past high bone turnover. Osteoclasts, when present, are usually seen within trabecular bone or at the small fraction of trabecular surface left without osteoid coating. With ABD, bone volume is frequently reduced. Reduced mineralization is coupled with a parallel decrease in bone formation. ABD is characterized by few osteoid seams (Fig. 3).

Mixed uremic osteodystrophy

Mixed uremic osteodystrophy (MUO) is caused primarily by defective mineralization with or without increased bone formation and by increased parathyroid hormone activity on bone, features that may coexist in varying degrees in different patients. Bone volume/tissue volume is extremely variable and depends on a dominant pathogenic cause. Other features include increased numbers of heterogeneous remodeling sites and usually an increase in osteoclasts. Active foci with numerous cells, peritrabecular fibrosis, and woven osteoid seams coexist with adjacent lamellar sites with a more reduced activity.

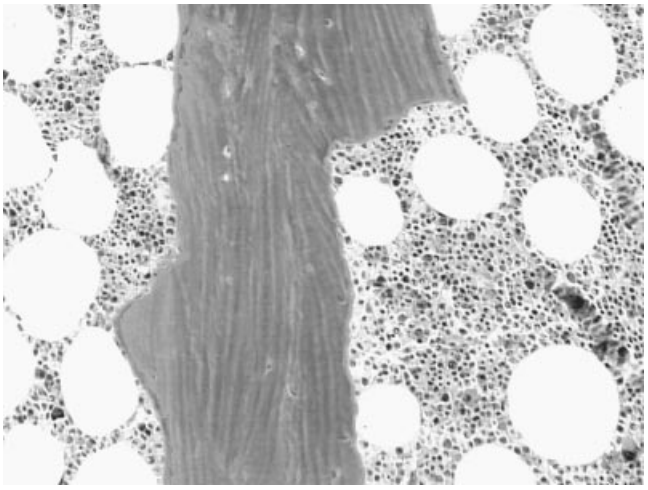

Fig. 3. Adynamic bone disease. No accumulation of osteoid. Absence of bone formation. Absence of active bone resorption. Undecalcified, 3 μm thick section of human iliac bone (modified Masson-Goldner stain, ×125 magnification).

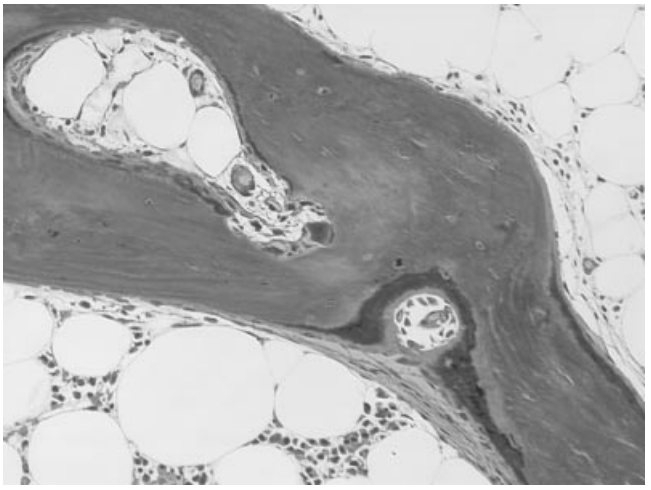

Fig. 4. Mixed uremic osteodystrophy. Increased fraction of trabecular surface covered by osteoid. Osteoid seams are wide or normal. Resorption lacunae with osteoclasts. Evidence of some peritrabecular fibrosis. Undecalcified, 3 μm thick section of human iliac bone (modified Masson-Goldner stain, ×125 magnification).

Therefore, greater production of lamellar or woven osteoid causes the accumulation of osteoid with normal or increased thickness of osteoid seams (Fig. 4). Although active mineralizing surfaces are often present in woven bone with higher mineralization rate and diffuse labeling, mineralization surfaces may be low in lamellar bone with a low mineral apposition rate.

Evolution of renal osteodystrophy

In the past, diversity in bone lesions was partly attributed to aluminum toxicity in bone. The major sources of contamination in end-stage renal disease (ESRD) patients are aluminum-containing phosphate binders and high aluminum content of water and dialysate. High morbidity and mortality accompany severe aluminum intoxication, and deferoxamine (DFO) chelation, though efficient, is not without risks. Therefore, efforts were made to develop tests capable of diagnosing this disease. Determining serum levels of aluminum before and after DFO treatment, alone or in association with serum PTH, proved useful, but only in those patients with high baseline serum aluminum levels who showed a marked increase after DFO challenge and had low serum PTH levels. Therefore, bone biopsies were the only unequivocal means to establish aluminum accumulation in bone and determine the type of renal osteodystrophy.

Today, aluminum dialysate content is under better control, and aluminum-containing phosphate binders have largely been replaced with calcium salts. To overcome parathyroid gland overactivity, therapeutic regimens ensuring better control of serum calcium and phosphorus levels and correction of calcitriol deficiency are also widely used.

In a previous study in which we analyzed the changing pattern of renal osteodystrophy in 2248 patients over 13 years (from 1983 to 1995), distribution of the four histologic forms varied [3]. MUO was found in the majority of patients over the years, but a slight decrease was seen in 1990 and 1995. PHBD was observed in a large number of patients in 1983, but decreased constantly until 1988, then increased gradually to reach a level in 1995 similar to the one found in 1983. LTOM incidence decreased progressively over the 13 years of the survey. The number of patients with ABD, which was first diagnosed in 1984 increased until the late 1980s, then stayed approximately the same. The trends for patients on hemodialysis and peritoneal dialysis were similar for the four histologic groups. However, peritoneal dialysis patients consistently exhibited more histologic signs of ABD and fewer signs of PHBD than did hemodialysis patients.

Between 1983 and 1984, the number of biopsied patients positive for aluminum increased from approximately 40% to approximately 60%, remained stable until 1992, then tended to decrease. In 1995, however, approximately 25% of the biopsies analyzed still showed aluminum in bone. Patients with low bone turnover, either LTOM or ABD, showed higher aluminum deposition than patients with other forms of renal osteodystrophy, and this did not change with time. More patients with stainable aluminum $> 30\%$ were in the LTOM, ABD, and MUO groups than in the PHBD group. From 1984 to 1995, bone aluminum accumulation decreased within each histologic group, and in 1995 aluminum accumulation was mostly seen in patients with MUO and ABD.

In spite of recent strategies, however, aluminum accumulation has not completely disappeared, and dialyzed

patients still present with PHBD, MUO, and ABD. Recent evidence shows that low bone turnover has both histologic and clinical relevance. Patients with this abnormality were found to have abnormal calcium homeostasis, higher incidence of fractures, more bone pain, delayed healing, and higher morbidity and mortality than did patients exhibiting other histologic abnormalities.

BONE BIOPSY TECHNIQUES

Prerequisites for bone biopsies: Bone labeling

The first prerequisite for an informative bone biopsy is proper *in vivo* labeling with specific, nontoxic, time-spaced bone markers before the biopsy. Antibiotics from the tetracycline family are used because they have spontaneous fluorescence and bind to actively forming bone surfaces. With labeling, the level of bone turnover and rate of bone formation can be determined, and possible mineralization abnormalities can be identified. A double labeling technique is best, although the schedule is somewhat more complicated for patients than one prolonged single administration. In most cases, the first label is administered for 2 days followed by an 8-to 15-day free interval. Anything fewer than 8 days lessens the distinction between labels, particularly in the case of a mineralization defect. Anything more than 15 days may artificially increase the number of single labels because the number of forming sites being completed or started will probably increase. During the 2–4 days after the free interval, the patient takes a second course of antibiotics. Bone biopsy is then performed 4–6 days after the last administration of tetracycline. During this delay, the tetracycline becomes slightly buried within mineralized osteoid and thus does not leach out. In an emergency, labeling can be shortened to a 1-day-on, 6-days-off and 1-day-on schedule with a single dose of oral tetracycline per day (1.0–1.5 g of tetracycline or 600–900 mg of Declomycin®). Gastrointestinal side effects may be greater with this approach. Using two labels with different colors assures accurate assessment of the mineralization rate. Tetracycline hydrochloride has a light yellow and demeclocycline hydrochloride (Declomycin®) a yellow-orange fluorescence. With the use of only one type of tetracycline, the two labels can merge in states of low bone turnover and be unrecognizable as a double label. For patients with normal renal function, dosages of tetracycline hydrochloride and Declomycin® are usually 500 mg and 300 mg t.i.d., respectively. For patients with impaired renal function, dosages should be reduced to 500 mg and 300 mg b.i.d.

Potential complications of bone biopsies

Complications from bone biopsies can include pain, hematoma, wound infection, and rarely neuropathy. However, studies show that horizontal or transiliac and vertical or superior biopsies of the anterior iliac crest result in very small morbidity and no mortality as a result of the procedure [4]. The operator's experience is important in minimizing morbidity and in obtaining an adequate specimen. Use of recently developed techniques should further decrease any complications. Patient reports of pain range from none to moderate and rarely severe.

Qualitative and quantitative evaluation of bone

Qualitative and quantitative evaluations of the bone sample constitute the final steps in processing a bone biopsy. Qualitative assessment consists of such factors as the suitability of a biopsy for morphometric analysis, the amount of sample needed, where histologic structures should be measured, and what elements to evaluate. With quantitative evaluation, numerical values are assigned to the various elements constituting bone [5]. Potential differences between groups of patients or normal individuals or changes occurring after treatment can then be statistically evaluated. Compared to earlier techniques for statistical evaluation, the semiautomatic computerized method introduced by Malluche et al [6] greatly reduced the time required to measure surface, thickness, volume, and profile count of bone. Software programs developed for use with the semiautomatic method are all reliable for calculating areas and counts, but some differences exist in computation of lengths, thus affecting all parameters. Advantages to the computerized-assisted histomorphometric analysis of bone also include many means of data verification, and the data obtained can be automatically transferred to any statistical software, thus avoiding entry error.

Fully automated image analyzers are available greatly reducing the time required to evaluate bone slides. Although they assess parameters of bone structure, they are not yet reliable in discriminating such elements as cellular details, detecting woven vs. lamellar bone, and recognizing erosion surface. However, complete automatic analysis of bone may be possible in the future, with improved video cameras, staining techniques, and computerized image-analysis capabilities.

CLINICAL APPLICATIONS

Nephrologists must determine a patient's level of bone turnover to apply the correct therapy. Serum PTH levels measured with radioimmunometric assay are commonly used to assess bone turnover in dialyzed patients. Although these levels have been found to be more sensitive than the previously employed radioimmunoassays, at present there is no consensus regarding the serum level of PTH that reflects normal bone turnover in ESRD patients. A recent study in our laboratory found that serum PTH levels between 65 and 450 pg/ml, seen in the

majority of dialysis patients, are not predictive of the underlying bone disease [7]. Because bone biopsies provide a sensitive measurement of bone changes, they more accurately determine the type of renal osteodystrophy and can indicate potential aluminum accumulation in dialyzed patients [8]. Bone biopsies also allow tailored therapeutic measures. The extent of aluminum deposits at the bone–osteoid interface and the level of bone turnover determine the optimal duration of chelation therapy.

If the biopsy shows no significant deposits of aluminum, the degree of bone turnover will help the practitioner determine the route, aggressiveness, and length of calcitriol therapy. Severe hyperparathyroidism with marked bone marrow fibrosis is an indication for high doses of intravenous calcitriol if the calcium phosphorus product can be controlled. A bone biopsy can predict whether there will be high resistance to intravenous calcitriol at the needed massive doses. In this case, parathyroidectomy may be necessary. The severity of the effect of secondary hyperparathyroidism on bone may also indicate the extent of the post-parathyroidectomy "hungry bone syndrome" and allow preventive measures such as the preoperative injection of calcitriol. In patients with mild to moderate increase in bone turnover with or without mineralization defect, doses of intravenous or preoperative calcitriol and duration of therapy may be adjusted to avoid the development of ABD. In case of ABD, calcitriol therapy is not desirable because of the risk of inducing hypercalcemia and extraosseous calcifications.

A bone biopsy establishes the precise relationships between serum indices of calcium metabolism and bone lesions. This enhances the interpretation of longitudinal follow-up of noninvasive parameters while the patient is undergoing a particular therapy.

MOLECULAR BONE HISTOLOGY

In situ hybridization histochemistry (ISHH) and immunohistochemistry (IHC) are two powerful methodologies used to investigate bone cell metabolism.

In situ hybridization histochemistry

Using complementary RNA probes labeled with a reporter molecule, ISHH can detect cellular nucleic acids, and therefore reveals cellular sources and differential gene expressions of specific biomolecules involved in bone metabolism, i.e., cytokines, matrix proteins, and receptor species. When applied to undecalcified bone section, ISHH can evaluate the relationship between gene expression and cell number and activity in the bone microenvironment.

Despite the invaluable information it provides, ISHH also has several limits that must be kept in mind when interpreting results. The sensitivity of ISHH is difficult to assess in different laboratories, and low levels of RNA gene expression may escape detection if the corresponding autoradiographic signal is dispersed over a large surface of the section or is masked by background. Therefore, caution must be used before important biological conclusions are drawn from negative results. Finally, specificity relies on the choice of suitable sequences of probe and stringency adopted for the hybridization procedure. Moreover, ISHH reveals only intracellular RNA transcripts and does not provide information about the subsequent steps of protein synthesis and extracellular secretion unless associated with immunohistochemistry.

Immunohistochemistry

IHC is highly complementary to ISHH in that it links the transcriptional and translational mechanisms of cells. IHC assesses the metabolic state of bone cells by visualizing protein production. Although the technique of IHC is well established in soft tissue, its use with hard tissue such as bone is relatively new. Therefore, researchers using IHC must be particularly careful to ensure a reliable and reproducible outcome.

Information derived from molecular histology

Researchers have demonstrated that factors believed to play important roles in regulating bone turnover are expressed in bone cells. ISHH has documented expression of bone matrix proteins, including type I collagen, osteocalcin, and osteopontin. These proteins are associated with cell-to-cell and cell-to-mineralized matrix adhesion. ISHH has also documented changes in biological activity measured by type I collagen and osteopontin mRNA expression in bone cells. The vitamin D receptor, which plays an important role in regulating bone turnover, has been shown to be expressed in bone cells through ISHH.

Recently, we used histomorphometric and ISHH techniques to document expressions of the cytokine interleukin-6 (IL-6) and its receptor IL-6R in renal osteodystrophy patients. IL-6 is believed to be a significant factor in bone remodeling. Our findings indicate that IL-6 and IL-6R are intricately involved in osteoclastic bone resorption [9].

Although molecular histology is still in its beginnings, its value has been clearly established. The continuing development of more efficient, economical methods will lead to its routine use as a morphological tool in the study of bone.

CONCLUSIONS

Bone biopsies are presently much more widely used for diagnosis and research than they have been in the past. However, traditional constraints continue to be perceived because of the procedure's invasiveness and cost,

potential pain for the patient, delays between the biopsy and pathology reports, lack of specialized centers with expertise to interpret bone samples, lack of technical training, and limited understanding of the information provided by the results.

Efforts to minimize these constraints have included improved instrument design and biopsy techniques and more intensive and detailed training of clinicians and pathologists. Advances in bone sample processing have resulted in faster turnaround time between bone biopsy and availability of histologic results. This has enhanced the value of bone biopsy in routine patient care. Also, bone morphometrists have adopted a uniform and simple nomenclature for bone histomorphometric parameters, and non-morphometrists can now better understand bone biopsy results. The establishment of the International Society for Bone Morphometry has increased the number of individuals involved in bone morphometry.

Alternatives to the bone biopsy continue to be pursued. The search for noninvasive serum or bone markers that predict bone turnover, mineralization status, bone aluminum accumulation, and cellular abnormalities has resulted in improved methods to determine serum levels of various calciotropic hormones, isolation of proteins and enzymes from bone, and development of commercially available assays. However, these alternatives have not proven to be specific or sensitive enough to effectively determine the potential value of a specific therapeutic regimen.

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