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Studies on EDTA Extracts and Collagenase Digests from Osteoporotic Cancellous Bone of the Femoral Head

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Using EDTA extraction and collagenase digestion, cancellous bone of the femoral heads from 10 normal and 9 osteoporotic subjects were analyzed for their contents of collagen, sialoprotein, proteoglycan and carbohydrate. The percentage of extracted matrix proteins of the osteoporotic bone in EDTA was significantly decreased, as was the collagenase-resistant fraction ($p < 0.05$). The sialic acid level in osteoporotic bone matrix was lower than in controls ($p < 0.05$).

The alterations found in bone matrix constituents in osteoporotic bone relative to controls suggest that in osteoporosis and fractures, not only bone mass changes, but also bone quality changes play a role in bone strength.

KEY WORDS: osteoporosis; femur head; bone matrix.

Osteoporosis is defined clinically as the loss of bone (osteopenia) sufficient to result in fractures with minimal trauma, generally of the spine but also of the hip. Pathophysiologically, osteoporosis is due to a predominance of bone resorption over bone formation. Unlike osteomalacia, there are no known specific biochemical abnormalities in osteoporosis and most authors agree that osteoporotic bone, while reduced in quantity, is essentially normal in chemical composition. The relative distribution of mineral and organic matters in bone, and particularly of collagen and noncollagenous proteins (NCP) in the organic fraction, play an important role in the maintenance of the biochemical proportions of bone (1, 2). Alteration in the transmission and distribution of stress in the joints may follow an alteration in the bones' chemical composition (3).

The purpose of the present study was to see whether or not biochemical alteration exists in the osteoporotic bone of the femoral head, which might explain its fragility.

Materials and methods

Nine osteoporotic femoral heads, five from women and four from men, were obtained at surgery from patients with femoral neck fracture. The mean age was 64 years (age range 58–76). Ten control human femoral heads, four from women and six from men, were obtained at necropsy as soon as possible after death. The mean age of the patients was 59 years (age range 54–68). Most of the patients died because of myocardial infarction and had no clinical or radiological signs of osteoporosis or osteoarthritis. These femoral heads were examined for gross morphology and those with obvious abnormality were discarded.

Bone preparation and extraction

Femoral heads were trimmed of cartilage and cortical bone. Only trabecular bone was used. It was cut in thin pieces, washed with cold water to remove blood material, defatted with trichlorethylene, and dried by immersion in ethanol–ether solution. A beater mill cooled by liquid nitrogen was used to powder bone. Only bone particles passing through a 100 mesh sieve were used in the bone demineralization procedure. This was carried out at 4°C with 100 g/L EDTA pH 7.5 for 10 days within a dialysis tube, according to the basic method of Herring (4). The dialysis tube had a cutoff of 3.500 kD.

In order to avoid proteolysis during extraction, the following protease inhibitors were used: 5 mmol/L benzamidine/HCl, 1 mmol/L phenylmethylsulfonylfluoride, 1 mg/L of soybean trypsin inhibitor, 5 mg/L of pepstatin A, 0.1 mol/L *N*-α-*p*-Tosyl-*L*-Lysine chloromethyl-ketone (TLCK), 0.1 mol/L *L*-1-Tosylamide-2-phenylchloromethylketone (TPCK).

After EDTA extraction and dialysis against water to remove EDTA, the total content of the dialysis tube was collected and referred to as bone organic matrix or bone matrix. Centrifugation at 2000 × *g* of this bone matrix gives a separation into EDTA-soluble matrix (EDTA extracts) and EDTA-insoluble matrix (EDTA residue). EDTA extracts and residues were lyophilized and stored frozen at −20°C until use.

Collagenase digestion

In order to explore bone matrix proteins remaining in EDTA residue, collagen was removed by digestion with bacterial collagenase-type CII (Sigma Chemical Co., St. Louis, MO 63178) at 37°C for 96 h as described by Herring (5). Phenylmercuric acetate was added as a bacteriostatic agent. The nondiffusible material that remained in the dialysis tube was referred to as the collagenase-resistant fraction (CRF), which includes the soluble collagenase-resistant fraction (SCRF) and the insoluble collagenase-resistant fraction (ICRF). The separation into SCRF and ICRF was performed by centrifugation (2000 × *g*).

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TABLE 1
Percentage Composition of Osteoporotic and Normal Femoral Head Bones after EDTA Demineralization and Collagenase Digestion of EDTA Residue

	Osteoporosis $n = 9$ Mean ± SD	Normal controls $n = 10$ Mean ± SD
EDTA extract (% dry bone powder)	0.83 ± 0.34[a]	1.13 ± 0.19
EDTA residue (% dry bone powder)	26.57 ± 4.16	24.13 ± 0.98
Bone matrix (% dry bone powder)	27.4 ± 4.5	25.26 ± 1.1
Soluble collagenase-resistant fraction (SCRF) (% dry EDTA residue)	4.85 ± 0.8[a]	3.64 ± 1.34
Insoluble collagenase-resistant fraction (ICRF) (% dry EDTA residue)	6.4 ± 3.4	4.7 ± 1.8
Collagenase-resistant fraction (% dry EDTA residue)	11.25 ± 4.2[a]	8.34 ± 1.4

[a] $p < 0.05$, Student's t test.

ANALYTICAL METHODS

Samples of EDTA extracts, EDTA residues, and collagenase digests were analyzed for their contents of collagen, sialoprotein, proteoglycans, and carbohydrate by measuring hydroxyproline (6), sialic acid (7), uronic acid (8), and hexoses (9), respectively.

The bone matrix content (x) was calculated according to the following formula:

$$x\ (\%) \text{ in bone matrix} = \frac{x \text{ in (EDTA extract)} + x \text{ in (EDTA residue)}}{\text{(EDTA extract + EDTA residue)}} \times 100,$$

x = component to be calculated,
( ) = total amounts of EDTA extracts or EDTA residues.

The moisture content of our bone samples was measured by drying bone at 105°C for 1 h, and was found to be about 9% for all samples tested.

STATISTICAL ANALYSIS

Student's t test was used for comparison of means from the two groups of bone studied. The difference was considered as significant when the probability was <0.05.

Each femoral head specimen was analyzed in triplicate and the mean value was used for calculating the mean in the group.

Results

Percentage compositions of osteoporotic and normal femoral head bone after demineralization with EDTA and collagenase digestion of the EDTA residue are presented in Table 1. The percentage extracted macromolecules in EDTA during demineralization of bone powder was significantly ($p < 0.05$) lower in the osteoporosis group compared to controls. The bone matrix, representing the total nondiffusible organic material of bone, and including both the soluble (EDTA extract) and insoluble (EDTA residue) macromolecules, was larger than in the control group but not significantly. After collagenase digestion a significantly higher amount of collagenase-resistant fraction was found in the osteoporotic samples compared to the control samples, suggesting a resistance to collagenase in osteoporosis.

The analytical results of bone matrix, EDTA extracts, and collagenase digests are summarized in Table 2. In bone matrix, the only difference between the two groups was in the sialic acid content, which was lower in the osteoporotic group ($p < 0.05$). The analytical results in EDTA extracts were similar in both groups. The collagenase digests (SCRF and ICRF) from the osteoporotic bone contained higher amounts of hexoses, sialic, and uronic acids ($p < 0.001$). The amount of hydroxyproline was also higher than the controls ($p < 0.001$) in the insoluble collagenase-resistant fraction of osteoporotic bone, but lower than the controls in the soluble collagenase-resistant fraction.

Discussion

Because there is no significant difference in bone composition between the sexes in animal studies (10) and in human studies (11), no distinction between sexes has been made in this study. This difference between sexes in relation to bone and osteoporosis is a question of bone quantity; men have more bone than women and lose less.

Given the variability of biochemical analyses of human bone, the results in the osteoporotic bone disclosed significant variations in percent EDTA extract, collagenase resistance, and sialic acid content of the matrix. This is in disagreement with the idea that osteoporotic bone, while reduced in quantity, is essentially normal in chemical composition.

The tissue extractability is often related to its age and turnover. The higher the extractability, the higher the turnover (11–16). In osteoporotic bone, the decrease in percent EDTA extract may reflect the decrease in the bone matrix turnover, as has already been suggested by Albright *et al.* in 1948 (17).

TABLE 2
Analytical Results in EDTA Extracts and Collagenase Digests from Osteoporotic and Normal Femoral Head Bones

	Bone matrix 100 g dry weight		EDTA extract 100 g dry weight	
	Osteoporosis	Controls	Osteoporosis	Controls
Hydroxyproline	12.94 ± 0.68	12.1 ± 0.64	1.23 ± 0.12	1.06 ± 0.70
Hexoses	0.45 ± 0.24	0.48 ± 0.02	2.0 ± 0.9	2.13 ± 0.06
Sialic acid	0.21 ± 0.08[a]	0.27 ± 0.01	1.80 ± 0.11	1.77 ± 0.03
Uronic acid	0.33 ± 0.18	0.26 ± 0.04	1.6 ± 0.9	1.64 ± 0.16
	SCRF 100 g dry weight		ICRF 100 g dry weight	
	Osteoporosis	Controls	Osteoporosis	Controls
Hydroxyproline	2.18 ± 0.26[b]	3.49 ± 0.96	8.23 ± 1.42[b]	3.63 ± 0.82
Hexoses	3.45 ± 0.65[b]	1.62 ± 0.36	1.83 ± 0.43[b]	0.43 ± 0.13
Sialic acid	2.3 ± 0.1[b]	1.55 ± 0.58	0.36 ± 0.05[b]	0.11 ± 0.05
Uronic acid	2.6 ± 0.6[b]	1.53 ± 0.44	0.69 ± 0.14[b]	0.15 ± 0.06

Results are expressed as g/100 g of dry weight of tissue. SCRF = soluble collagenase resistant fraction. ICRF = insoluble collagenase resistant fraction. Values are means ± SD for 10 controls and 9 osteoporotic femoral heads.
[a] $p < 0.05$.
[b] $p < 0.001$.

Noteworthy also is the decrease of sialic acid levels in osteoporotic bone matrix as shown by the present study. The role of bone sialoprotein (BSP) is not yet known. This structural glycoprotein is more concentrated in mineralized tissue (4) as are some plasma proteins (18), and it has a great affinity for minerals, thus suggesting its role in the mineralization process. The lowest level of sialic acid was also found by Quelch *et al.* (19) in osteoporotic bone. The decrease of BSP may thus be interpreted as an index of the decrease of bone mineralization in osteoporosis. However, this deserves experimental confirmation. The collagenase resistance of the osteoporotic bone suggests that osteoporotic bone collagen is more mature and cross-linked, or that this collagen is particularly denatured, both conditions which may increase collagenase resistance (20). However, no such evidence of collagen alteration has been reported.

The apparently contradictory finding of increased sialoprotein in the collagenase-resistant fraction and decreased amount in total bone matrix could be due to the difference in relative distribution of the sialic acid in the different portions of bone matrix examined. Because EDTA extracts + soluble collagenase fraction and insoluble collagenase fraction represent only a small part, ± 10% of the bone matrix left after collagenase, it is possible that in that small fraction sialic acid is more concentrated in osteoporosis, while in the total matrix the level is lower.

The relative changes, qualitatively and quantitatively, of collagenous and noncollagenous proteins of bone matrix are not unique for osteoporosis. In a previous study (21), we found a significantly increased amount of hexoses and uronic acid in bone matrix, and an increased amount of total EDTA extract and sialoprotein in EDTA extract in cases with primary osteoarthritis. Since there is an inverse relationship between osteoarthritis and osteoporosis, the finding of biochemically opposite changes in bone matrix in these two conditions suggests a different pathophysiological bone diathesis in these conditions. In line with this hypothesis are the findings of Ghosh *et al.* (1) that alterations of the relative distribution of collagen to noncollagenous matrix components parallels biomechanical function of disks in dogs.

This parallelism between biomechanically different functions and biochemical differences in bone matrix proteins was also observed in the analysis of bone matrix constituents from different anatomic sites, such as femur, iliac crest, and ribs (22).

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