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In Vitro Synthesis of Normal Bone (Type I) Collagen by Bones of Paget's Disease Patients (40812)[1]

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Paget's disease of bone is a common disorder of man, characterized by localized skeletal deformity, both grossly and microscopically, by a marked increase in the number of bone cells, and by characteristic inclusions in the nucleus and sometimes cytoplasm of osteoclasts. Because of a high incidence of associated connective tissue abnormalities such as medial arteriosclerosis and angioid streaks of the retina (defects in Bruch's membrane) and because of a significant familial clustering of the disease, it has been postulated that the disease may result from an inborn error of collagen biosynthesis (1).

Krane and colleagues have studied the amino acid and glycosylated hydroxylysine content of bone from patients with Paget's disease and found no significant differences from normal (2). Misra has investigated collagen crosslinks in autopsy specimens of Pagetic bone and found evidence of abnormal crosslinks (3). Rarely, Paget's disease has been associated with pseudoxanthoma elasticum (4). Francis and Smith found that in Paget's disease of bone the amount and stability of the polymeric collagen fraction extracted from the skin is significantly reduced when compared to age-matched controls (5). So far, no studies of the type of collagen present in Pagetic bone have been reported.

In this report we present data concerning the type of collagen synthesized *in vitro* by bones obtained at surgery from patients with Paget's disease. Following the isolation of the α_1 chains of collagen synthesized by normal and Pagetic bone, their CNBr peptide patterns were investigated. In each instance, Type I collagen was the only molecular species synthesized by both normal and Pagetic bone. In addition, it was found that the amount of collagen synthesized by Pagetic bone was greater than that synthesized by normal bone.

Materials and methods. All culturing medium such as Dulbecco's modified eagle medium (DMEM) *with high glucose*, fetal calf serum and penicillin–streptomycin (P/S) solutions were obtained from Grand Island Biological Company. Pepsin (PM grade) was purchased from Worthington Biochemicals. Reagents for sodium dodecyl sulfate (Na Dod SO_4) electrophoresis were purchased from Bio-Rad Laboratory. Aquasol and [2,3-^{3}H]proline (30–50 Ci/mmol) were obtained from New England Nuclear. Standard radioactive collagen α_1(I) and α_2(II) chains were kind gifts of Dr. Paul Benya.

Collagen labeling and purification. Both "involved" and "uninvolved" bones were obtained from five Paget's patients at surgery. Both light microscopy and electron microscopy were used to define whether the bone was normal or typical of Paget's disease. Control bone samples were removed from a normal 18-year-old man who died following an automobile accident. The bones were broken into small chips with a bone clipper, cleansed, and washed extensively with DMEM to remove any ad-

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herent soft tissue. The bone chips were then radioactively labeled for 48 hr in 15 ml culturing medium (DMEM and 20% fetal calf serum) containing [^{3}H]proline (100 μCi/mmol), BAPN (125 μg/ml), and L-ascorbic acid (50 μg/ml). At the end of the incubation period, the bone chips were separated from the medium and powdered in a liquid nitrogen-cooled freezer/mill.

Following demineralization with 0.5 *M* ethylene diaminetetraacetic acid (EDTA), pH 8.0, the powdered bone was added back to the original medium and dialyzed against 0.5 *N* acetic acid twice overnight. The mixture was digested by shaking for 48 hr at 4°C with pepsin (2 mg/ml) and lyophilized. The pepsin was inactivated by dissolving the lyophilized sample in 10 ml cold solution of 1 *M* NaCl–50 mm Tris–HCl, pH 7.5. The suspension was titrated to neutrality with 2 *M* NaOH solution and dialyzed against 1 *M* NaCl–50 mm Tris–HCl, pH 7.5 at 4°C overnight. Any insoluble material was removed by centrifugation at 10,000*g* for 1 hr. The radioactive collagen was purified by neutral and acid salt precipitation (6). The collagen precipitate was dissolved and dialyzed extensively against 0.5 *M* acetic acid. The final product was used for Na Dod SO_4 electrophoresis and CMC chromatography.

Samples of pepsin-soluble collagen and the insoluble residue of bones of Paget's patients and the normal control were hydrolyzed with 6 *N* hydrochloric acid at 108°C overnight; the collagen content was estimated by hydroxyproline assay (7). The total collagen content of both bone samples, therefore, represents the sum of the soluble and insoluble fractions.

The radioactive collagen was further purified by DEAE column (0.9 × 8 cm) chromatography (8) to remove any contaminating proteoglycans. The purified material was dialyzed against 0.5 *M* acetic acid and then against CMC running buffer (60 m*M* Na-acetate, 1 *M* urea, pH 4.8). The collagen α chains were separated by a modified CMC chromatograph procedure (8). The α chains were dialyzed against 0.5 *N* acetic acid, lyophilized, and stored for CNBr peptide analysis.

Samples of purified radioactive collagen and collagen peptide products of CNBr cleavage were subjected to Na Dod SO_4 electrophoresis, the details of which have been previously published (6, 9, 10). A continuous Tris–Borate buffer system was used. Fractionation of collagen chains was carried out on 5% gels, while 15% gels were used to analyze the CNBr peptides that originated from CMC-isolated α chains. Radioactive gels were cut into 1-mm slices, hydrolyzed in 0.2 ml of 0.4 *M* NaOH at 55°C overnight, and counted in acidified aquasol (11).

CNBr cleavage of α chains was performed under nitrogen for 4 hr at 30°C with two- to fourfold (w/w) excess of CNBr (10 mg/ml in 70% formic acid) (12).

After decalcification, but before pepsin treatment, an aliquot was taken from each total bone and medium mixture of normal, involved, and uninvolved Pagetic bone. The aliquots were dialyzed extensively against a 1% NaCl, 50 m*M* Tris–HCl (pH 7.5) solution. The nondialyzable counts divided by the weight of bone sample were used to calculate total protein production per milligram of bone. Collagen synthesis was estimated by analysis of [^{3}H]hydroxyproline after acid hydrolysis and separated on the 50-cm column of a JEOL-5 AH amino acid analyzer.

Results. By light microscopy, the typical histologic features of Paget's disease were found in the bone specimens thought to be involved with Paget's disease. Electron microscopy revealed typical nuclear inclusions in the nuclei of osteoclasts in each specimen shown to be abnormal by light microscopy (data not shown).

Over 60% of the total collagen present in bone samples of Paget's disease patients and the normal control were solubilized by pepsin (Table I). The nature of collagen

TABLE I. PERCENTAGE OF COLLAGEN IN PAGETIC AND NORMAL BONE SOLUBILIZED BY PEPSIN

Tissue	$\frac{\text{Pepsin soluble collagen}}{\text{Total collagen}} \times 100$
Pagetic bones ($N = 5$: $\bar{X} \pm$ SEM)	64.3 ± 11.2
Normal bone ($N = 1$)	71.2

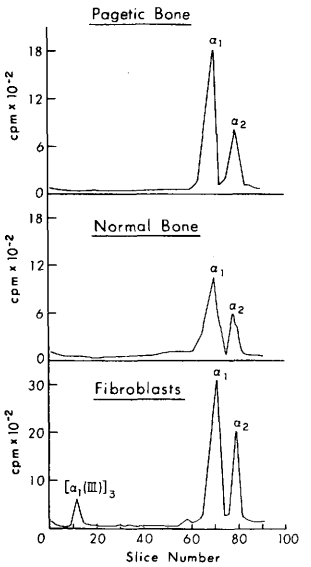

FIG. 1. Sodium dodecyl sulfate electrophoretic patterns of collagen synthesized by Pagetic bones, normal bones, and human fibroblasts.

produced by Pagetic, normal, and skin fibroblasts was estimated by Na Dod SO_4 electrophoresis (Fig. 1). Both Pagetic and normal bones produced α_1 and α_2 with a ratio of approximately 2.0 to 1, reflecting Type I collagen production. On the other hand, human skin fibroblasts synthesized both Type I (α_1:α_2 = 2:1) and Type III, $[\alpha_1(III)]_3$, the latter being sensitive to reduction with mercaptoethanol. In this case, Type I collagen made up 92% of the total collagen production, the rest being Type III.

To further characterize the radioactive synthesized collagen of Pagetic bone, it was fractionated using CMC chromatography (Fig. 2). The α_1 to α_2 ratio was approximately 2 to 1, confirming the Na Dod SO_4 electrophoresis data. The α_1 was collected and then subjected to CNBr cleavage. The resulting peptides were analyzed by 15% Na Dod SO_4 gel electrophoresis. Standard α_1(I) and α_1(II) chains were treated in an identical fashion and used for comparison (Fig. 3). The CNBr data conclusively showed that Pagetic bone α_1 chain was α_1(I) rather than α_1(II). α_1(I)CB_6, used as a marker peptide for Type I collagen, appears as a permanent peak in Pagetic bone as well as in normal bone (not shown) after treatment of the isolated α_1 chains with CNBr. No Type II collagen is present, as indicated by the absence of α_1(II)CB10, 5, a characteristic marker for this type of cartilage associated collagen.

Figure 4 shows the spectrophotometric scan and picture of the Comassie blue stained gel of CNBr peptides of α_1 chain of Pagetic bone. It further illustrates that the α_1 chain of Paget's bone is identical to α_1 chain of Type I collagen.

Figure 5 summarizes the total protein as well as collagen synthesized by Pagetic (involved and uninvolved areas) and normal bones. Involved Pagetic bones were the

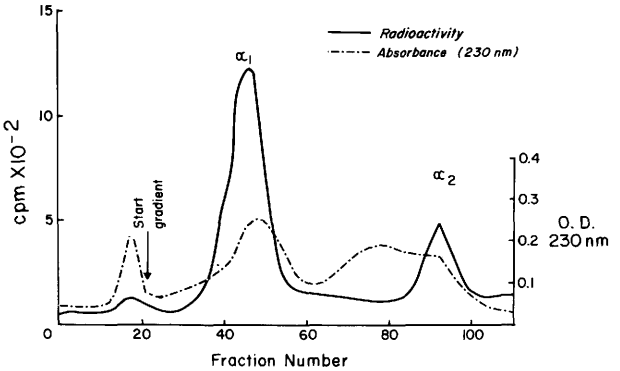

FIG. 2. Carboxymethyl cellulose chromatography of collagen from Pagetic bones. Purified radioactive collagen was fractionated on a 1.6 × 9-cm column of carboxymethyl cellulose with a 500-ml linear gradient (0.0 to 0.1 *M* NaCl in 1 *M* urea, 60 m*M* sodium acetate, pH 4.8). The volume of each fraction was 2.5 ml, and 100 μl was used for scintillation counting. Ultraviolet absorption was monitored at 230 nm.

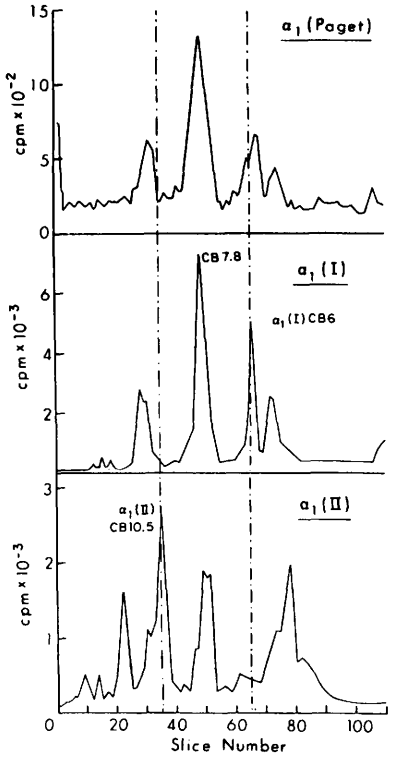

FIG. 3. Comparison of cyanogen bromide peptide profiles of α_1 chains obtained from collagen of Pagetic bones, α_1(I) from human fibroblasts, and α_1(II) from rabbit articular cartilage.

most metabolically active. They made four time more protein and sixfold more collagen than normal controls.

Discussion. At least four genetically distinct species of collagen have been identified in vertebrate tissues (13–19); each differs from the other in its primary structure (amino acid sequence of the subunit polypeptide chains). Type I collagen consists of two different α_1 chains, designated as α_1(I) and α_2 and its molecular structure is $[\alpha_1(I)]_2\alpha_2$. The other three types of collagen are composed of three identical α chains designated as $[\alpha_1(II)]_3$, $[\alpha_1(III)]_3$, and $[\alpha_1(IV)]_4$ (20).

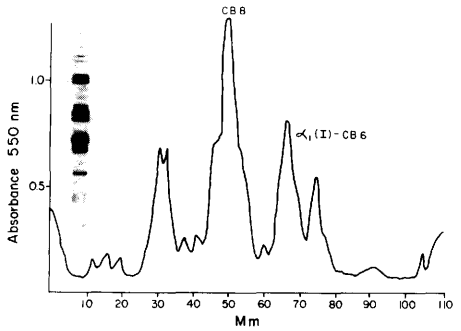

FIG. 4. Spectrophotometric scans of the Comassie blue stain gel of α_1 chain of Pagetic bones.

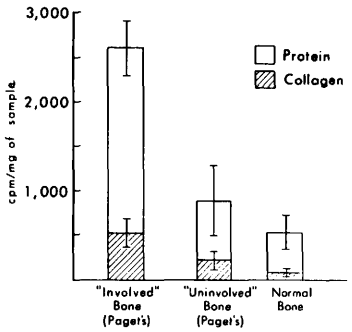

FIG. 5. Total protein and collagen synthesis by Pagetic and normal bone.

Collagen accounts for roughly 20% of the dry weight and 90–95% of the organic matrix of bone (21, 22). In animals and human bones examined thus far, only Type I collagen has been found (23–26). In our present study, we attempted to detect biosynthesis of genetically distinct types of collagen by Pagetic bones. We radioactively labeled both Pagetic and normal bones *in vitro*, then characterized the collagen so synthesized with established biochemical techniques such as Na Dod SO_4 gel electrophoresis, CMC chromatography, and CNBr peptide analysis.

Both Na Dod SO_4 gel electrophoresis and CMC chromatography technique showed that Pagetic and normal bones synthesized only Type I collagen (α_1:α_2, 2:1). Cyanogen bromide peptide analysis of the radioactive α_1 chains further established that both Pagetic and normal bone produced identical α_1(I) chains, thus ruling out any possibility of presence of α_1(II) cartilage collagen and α_1(III) collagen.

Pagetic bones are highly cellular, a number of cell types including fibroblasts can be seen under the microscope. The possibility that the collagen produced by the Pagetic bones that we examined is due to fibroblasts has been suggested. Although

this possibility cannot be ruled out completely, we consider that it is not a significant factor for the following reasons: (i) we meticulously removed all the soft tissues surrounding the bone chips, then washed and flushed them with copious amounts of culture medium (DMEM) extensively to remove any loose adhering cells; (ii) no synthesis of Type III collagen was detected by our Na Dod SO_4 gel electrophoresis and CMC chromatography (Figs. 1 and 2). Since 10–15% of the collagen produced by fibroblasts are Type III, some Type III collagen should be evident if there is any significant production of collagen by contaminating fibroblasts; (iii) we examined the pepsin soluble collagen of Pagetic and normal bone chemically (Figs. 2 and 4). Again, only Type I collagen was found. These collagens are already present in the bones and are not collagen synthesized in the 24-hour labeling period; and (iv) Krane *et al.* (2) have examined the amino acid composition of collagen isolated from Pagetic and normal bones and found no differences.

The higher metabolic rate of collagen and protein synthesis of Pagetic bones was interesting but not surprising. Since Pagetic bones are highly cellular as compared to normal bones, the increased protein production per milligram of bones should be higher.

In conclusion, we presented evidence that bones from patients with Paget's disease contain Type I collagen which is identical to Type I collagen from bones of normal individuals, and that the bones from patients with Paget's disease do not contain Type II or III collagen. However, our present methods do not rule out the possibility of synthesis of a small amount of Type IV or Type A–B collagen by Pagetic and normal bone. Questions concerning the fidelity of processing of procollagen, the pattern of crosslinking, and synthesis of Type IV or Type A–B collagen remain to be answered.

Summary. Bone samples were obtained from five Paget's disease patients and one normal individual at surgery. We characterized the collagen content of bone samples chemically, using sodium dodecyl sulfate gel electrophoresis and cyanogen bromide peptide analysis, and also determined the type of collagen produced *in vitro* by these samples. Both Pagetic and normal bones make normal bone collagen with the α_1 to α_2 chains ratio equal to 2:1. However, Pagetic bones synthesized three to four times more protein and collagen per milligram of tissue as compared to normal bones.

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