J. Biochem. 122, 109-115 (1997)

Glycosylation of Human Bone Collagen I in Relation to Lysylhydroxylation and Fibril Diameter[1]

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Received for publication, February 7, 1997

Posttranslational modifications (lysylhydroxylation, glycosylation, and crosslink formation) of collagen I and the trabecular bone volume (TBV) as well as the supramolecular organization of human vertebrae were studied by analyzing vertebral bones of 55 individuals (22-93 years of age). The degree of lysylhydroxylation of both α-chains of collagen I showed a significant inverse correlation with the TBV, while only a weak correlation between lysylhydroxylation of α2(I) and the age of the donor was observed. The degree of glycosylation of collagen I was significantly correlated with both the level of lysylhydroxylation and the degree of osteopenia. Electronmicroscopic evaluation did not show any relationship between the level of collagen glycosylation and the diameter of *in vivo* formed fibrils or *in vitro* formed fibrillar aggregates. In our study the molar ratio of the mature collagen crosslinks, pyridinoline and deoxypyridinoline, showed a slight tendency to be higher, in particular in the samples with a high level of lysylhydroxylation. This ratio was recently found to be significantly increased in avian osteoporotic bone. Our data suggest that the increased level of lysylhydroxylation in human osteopenia is related to the glycosylation of collagen I, while it seems to have little impact on the formation of the mature, non-reducible collagen crosslinks investigated. Based on our observations it appears unlikely that the different diameters of collagen fibrils contribute greatly to the reduced biomechanical stability reported for overhydroxylated, osteopenic bone tissue.

Key words: bone, collagen, modification, osteopenia.

Collagen I as the major structural component of bone provides the scaffold for mineralization and is responsible for the high tensile strength of bone tissue. Any defect in the collagenous structure could therefore adversely affect the functional integrity of bone and increase its fragility. Furthermore, an altered collagenous bone matrix may be either the result of or the trigger for disturbance of bone cell function (*1*).

Osteoporosis is characterized by losses of bone mass and of the trabecular microarchitecture, both of which lead to an increased risk of fractures (*2*). While these morphological changes were known for sometime on the microscopical level, only recently has there been increasing evidence that histostructural changes, as observed in osteopenic bone disease, are associated with characteristic alterations of the collagenous matrix, *e.g.* increased hydroxylation of lysyl residues in collagen I in man and chicken (*3-5*). This posttranslational modification is crucial since hydroxylysine residues represent the attachment site for glycosides, and provide the basis for the formation of intra- and intermolecular crosslinks (*6*). Furthermore, there is increasing experimental evidence that an elevated degree of lysylhydroxylation is correlated with the reduced biomechanical stability of the respective bone tissue (*5, 7*). It is still open to debate whether or not the biochemical changes observed affect the supramolecular organization (*e.g.* fibril formation or fiber aggregation) of the collagenous bone matrix. It is interesting to note that at the regulatory level, osteoanabolic transforming growth factor-β (TGF-β) apparently counteracts lysyl overhydroxylation by downregulating the steady state level of lysylhydroxylase mRNA (*8*).

In the present study we examined to what extent lysylhydroxylation modulates downstream events of procollagen processing such as glycosylation of hydroxylysine residues, and the formation of the mature collagen crosslinks, pyridinoline (PYD) and deoxypyridinoline (DPD). For the first time we systematically addressed the question of whether or not posttranslational modifications of collagen I in human bone have an impact on the diameter of collagen fibrils: the morphology of *in vitro* formed fibrillar aggregates of human bone collagen as well as that of isolated fibrils formed *in vivo* was investigated by electron microscopy and the relationship to posttranslational modifications of collagen I was examined.

MATERIALS AND METHODS

Tissue Sampling and Preparation—Vertebral trabecular

[1] This study was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 367/A1) and BMFT grant no. 01 KM 9303/8.

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bone from 55 patients (28 females, 27 males), 22-93 years of age, was analyzed in this study. The patients were randomly selected, although we intentionally included patients of different ages and with apparent differences in bone densities on macroscopic inspection. Patients with endocrinopathies, connective tissue disorders, metastatic bone disease, or systemic inflammatory diseases were excluded from the study. All clinical diagnoses and presumed causes of death were verified by autopsy. Thirteen patients had a history of osteoporosis (10 females, 3 males), including 8 with established osteoporosis (one or more vertebral fractures or a fracture of the femoral neck; 7 females, 1 male). Trabecular bone specimens from the thoracal and lumbar spine (TH5-L5) were obtained at autopsy within 36 h after death. Using an electric band saw, cortical bone, intervertebral discs, and adherent connective tissue were carefully removed. The remaining trabecular bone was frozen in liquid nitrogen and then cut into small cubes (0.5-1.0 cm^3). Samples of the second lumbar vertebral body were taken for morphometric analysis. The small cubes were extensively washed in distilled water and 96% ethanol to remove blood and residual fat. Using a stainless steel homogenizer and a conventional mortar, the bone samples were powdered under liquid nitrogen. The bone powder (1.5-16 g dry weight) was demineralized by dialysis at 4°C against repeated changes of 0.4 M EDTA (pH 7.4) for 5 weeks and then against 0.05% acetic acid for an additional 3 days. All dialysis steps were carried out in the presence of phenylmethansulfonylfluoride (3 mg/liter) as a protease inhibitor.

Morphometric Analysis—Morphometric analysis was performed on the central part of the second lumbar vertebral body. Pieces of approximately 0.4 cm^3 were fixed for 48 h in 10% formaldehyde and then dehydrated with increasing concentrations of ethanol. The samples were then embedded at −14°C for 24 h in a 2:3 mixture of methylmethacrylate and butylmethylacrylate. Four micrometer undecalcified sections were stained according to the Masson-Goldner trichrome method. Morphometric analysis was performed at a magnification ×160 using a ZEISS universal microscope (ZEISS, Oberkochem, Germany) connected to a camera. Each histological picture was digitized and analyzed using morphometry computer software (Optoquant; Computer & Vision, Lübeck, Germany), so the trabecular bone volume (%TBV) and the trabecular perimeters were determined automatically (*9*).

Quantitation of Proteins—A 20 mg aliqout of the demineralized bone powder was dissolved in 0.5 ml of 0.3 M KOH by heating to 70°C for 1 h. The protein content was determined according to Lowry *et al.* using bovine serum albumin as a standard (*10*).

Extraction and Separation of Collagen I—Limited pepsin digestion was performed to solubilize bone collagen. Demineralized bone samples were stirred in a pepsin solution at 4°C for 24 h (0.1 mg/ml in 0.2 M sodium acetate, pH 1.5; Boehringer Mannheim, Germany). After centrifugation at 200,000×g (1 h, 4°C), the supernatants were neutralized and stored at −20°C. This digestion procedure was repeated five times and all the neutralized supernatants were pooled.

In order to isolate collagen I, sequential neutral salt precipitations were performed by dialysis against 1.8 and 2.5 M NaCl-solutions (0.05 M Tris, pH 7.4) (*5*). After centrifugation (200,000×g, 1 h, 4°C), the pellets were dissolved in 0.05% acetic acid and then dialyzed against the same solvent to remove residual salt. Aliquots of the total pepsin extracts and of the salt precipitate of each tissue sample were lyophilized and then redissolved in SDS-sample buffer. Visualization of the extracted collagens and the isolated collagen I was perfomed by SDS-PAGE as described previously (*11*).

Isolation of α1(I) and α2(I)—Aliquots of the collagen I-containing 2.5 M NaCl precipitates were lyophilized and dissolved in 0.5 ml 4 M guanidine hydrochloride, heat denatured at 56°C for 10 min, and then immediately loaded onto a Waters C18 reverse phase column (*11, 12*). The column was developed with a linear gradient of 21-32% of acetonitrile/0.1% trifluoroacetic acid (solvent B) in 0.1% (v/v) trifluoroacetic acid in water (solvent A) over 37 min at the flow rate of 1 ml/min. Individual fractions were analyzed by SDS-PAGE.

Amino Acid Analysis—To determine the degree of lysylhydroxylation, 100 μg aliquots of lyophilized α1(I) and α2(I) chains were hydrolyzed for 24 h at 110°C with 6 M HCl and 0.1 mercaptoethanol under nitrogen. For the determination of galactosyl- and glucosyl-galactosyl-hydroxylysine residues, 100 μg aliquots of the lyophilized 2.5 M NaCl precipitate were hydrolyzed for 24 h at 110°C with 2 M KOH. Purification was performed by cation exchange chromatography on Dowex 50W-X8 as described by Tenni *et al.* (*13*). The eluates were lyophilized, dissolved in the sample buffer for amino acid analysis, and then separated with an amino acid analyzer using a step gradient composed of 7.3 ml buffer Na-F and 3.2 ml Na-D (Beckmann, Germany) at the column temperature of 77°C and the flow rate of 20 ml/h. A glucosylgalactosylhydroxylysine standard was prepared in the laboratory from a marine sponge, and used for the quantification of glucosylgalactosylhydroxylysine (GGH) and galactosylhydroxylysine (GH; according to Ref. *13*). Hydroxylysine (Hyl) and lysine (Lys) were commercially obtained from Serva. The degrees of lysylhydroxylation and glycosylation were expressed as the ratios of Hyl/(Hyl+Lys)×1,000 and GH+GGH/(GH+GGH+Hyl), respectively. The restricted amounts of bone samples available only allowed determination of the degree of glycosylation in 12 cases. The high precision and accuracy of the assay had been documented previously (*8*).

Determination of Mature Collagen Crosslinks—The determination of pyridinoline (PYD) and deoxypyridinoline (DPD) in bone tissue was described in detail in a recent paper (*14*). Briefly, demineralized bone samples (10 mg) were dissolved in 6 N hydrochloric acid and then hydrolyzed at 110°C for 24 h. After filtration and evaporation to dryness at 50°C each hydrolysate was dissolved in *n*-butan-(1)-ol-glacial acetic, mixed with a cellulose powder (CF 1) slurry, loaded on a glass filter, and then washed with the mobile phase solvent. The pyridinium-containing fraction was eluted with water. This adsorption chromatography (CF 1) was followed by molecular sieve chromatography (Sephadex G-10). The enriched pyridinium-containing fractions were collected for the subsequent preparative reversed-phase HPLC using a two-pump gradient system (Model M 480; Gynkotek, Germering, Germany) equipped with a Gynkotek RF 1002 fluorescence monitor. The column was developed with a two-step gradient of 5-27% (5 min) and 27-31% (15 min) with 0.22% (v/v) heptafluorobutyric

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acid (HBFA) in 80% aequeous acetonitrile (solvent B) in 0.22% (v/v) HBFA in water (solvent A). The eluate was monitored for PYD and DPD fluorescence (excitation at 279 nm, emission at 397 nm). The tissue concentrations of PYD and DPD were expressed as both nmol per mg dry weight and nmol per mg protein. We have previously shown that the age of the donor and the degree of osteopenia do not influence the protein content of the bone tissue (*5*). The limited amounts of bone samples available only allowed determination of the tissue concentrations of PYD and DPD in 36 cases.

Statistical Analysis—Double measurements of each of two aliquots from each sample were performed. Regression analyses were performed according to Ref. *15*.

Investigation of Bone Collagen Fibrils Formed In Vivo (16)—Using a stainless steel homogenizer and a conventional mortar, non-decalcified bone samples were powdered under liquid nitrogen. The powder was resuspended in 4 ml of ultra-pure water. The samples were totally homogenized and then sonicated for 2 min until a milky suspension was obtained. A 10 μl aliquot of the total material was drop-drained on to a carbon-coated copper 600-mesh grid, washed twice with distilled water, and then air-dried. Unstained grids were examined by electron microscopy using a ZEISS EM 109 electron microscope at an accelerating voltage of 100 kV. Images were recorded between $\times$5,000 and $\times$30,000 magnification (*16*). To determine the average fibril diameter, 200 fibrils per sample were measured.

In Vitro Fibril Formation—Stock solutions were prepared by ultracentrifugation of collagen solutions of an original concentration of 1 mg/ml (200,000$\times g$, 1 h, 4°C). The upper half of the supernatant (monomeric collagen) was withdrawn and adjusted to a final concentration of 200 μg/ml, which was judged spectropolarimetrically (Jasco J-500A). The self-assembly conditions followed a modification of the method described by Williams *et al.* (*17*). Samples were then transferred to a thermo-controlled Gilford cuvette. Fibril formation was triggered by increasing the temperature to the incubation temperature of 34°C. After 1,000 min, 4$\times$3 μl were transferred to formvar-coated copper grids using a micropipette with a widened tip diameter. The fibrils were allowed to settle for 30 min. Then the buffer was drained off cautiously and three washing steps were performed. Subsequently, the fibrils were stained with freshly prepared phosphotungstate (1%, pH 7.4) for 2 min, washed another 3 times, and then dried. The grids were examined under a Zeiss EM 109 electronmicroscope. The average fibril width was determined for 200 fibrils per sample using the morphometry computer software mentioned above.

RESULTS

Morphometric Analysis—The mean trabecular bone volume (TBV) was 9.03 +/−2.31 in the entire study group, ranging from 3.1 to 14.8%. As expected from previous studies, the TBV significantly decreased with age ($n=55$, $r=-0.447$, $p<0.001$; data not shown; *9*).

Collagen Solubility—As described previously, the yield of soluble collagen on limited pepsin digestion was 56% of the total demineralized material, without any difference between normal and osteopenic tissue (*5*).

Lysylhydroxylation and Glycosylation of Lysyl Residues—The data obtained for samples of human vertebral trabecular bone ($n=55$, 28 females, 27 males) confirmed the highly significant negative correlation between the TBV and the degree of lysylhydroxylation (LysOH) of the α2-chain of collagen I (Fig. 1a; $r=-0.56$, $p<0.001$). In addition, a similar negative correlation was found for the α1-chain of collagen I (Fig. 1b; $r=-0.38$, $p<0.01$). In contrast, there was only a weak correlation between LysOH of either α-chain and the age of the donor [$r=0.30$, $p<0.05$; values for α2(I)]. The mean lysylhydroxylation was slightly lower in males than in females [173+/−21 *vs.* 185+/−19 for α2(I) and 86+/−20 *vs.* 95+/−18 for α1(I), respectively; the values being expressed as Hyl/(Lys+Hyl)$\times$1,000].

Investigating the following step of posttranslational modification, we found that the attachment of GH and GGH to hydroxylysine residues of total collagen I significantly increased with decreasing TBV (Fig. 2; $n=12$, $r=-0.75$, $p<0.005$) and showed a close correlation with the degree of lysylhydroxylation ($n=12$, $r=0.66$, $p<0.01$; Fig. 2b). Taking into account the known numbers of lysine residues in the α1(I)- and α2(I)-chains (SWISS-PROT 23 Database, Intelli Genetics), the number of glycosylated Hyl residues can be calculated to range from 0.57 to 1.99 per single

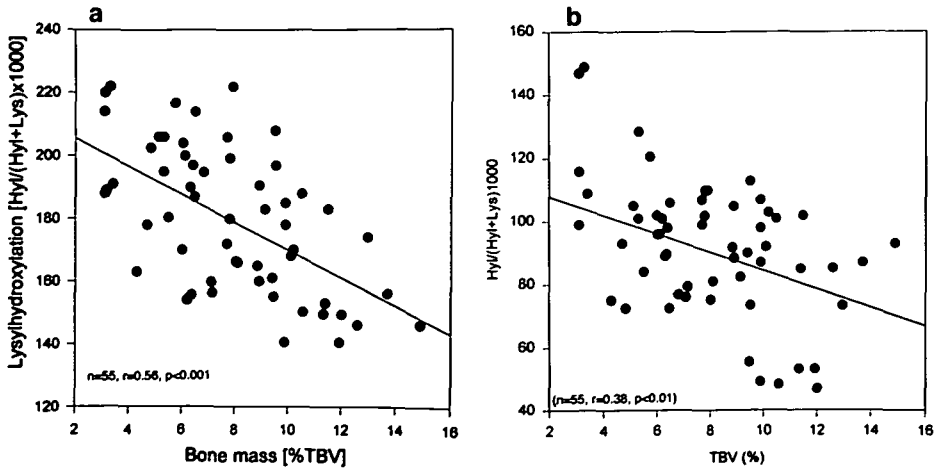

Fig. 1. **a: Negative correlation between the degree of lysylhydroxylation of α2(I) [expressed as Hyl/(Hyl+Lys)$\times$1,000] and the trabecular bone volume (expressed as % TBV). $n=55$, $r=-0.56$, $p<0.001$. b: Negative correlation between the degree of lysylhydroxylation of α1(I) [expressed as Hyl/(Hyl+Lys)$\times$1,000] and the trabecular bone volume (expressed as % TBV). $n=55$, $r=-0.38$, $p<0.01$.**

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collagen molecule in our specimens.

The relative proportion of diglycosides (GGH) compared to monoglycosides (GH) showed a slight tendency to be higher with increasing TBV ($n=12$, $r=0.45$, n.s.).

Collagen Crosslinks—Analysis of the hydroxylysine-derived mature crosslink components, pyridinoline (PYD) and deoxypyridinoline (DPD), did not reveal any significant relationship beween the TBV and the concentration of either PYD and DPD in the bone tissue. Likewise, the molar ratio of PYD and DPD was unrelated to the TBV, and there was no significant elevation of the PYD/DPD ratio with increasing lysylhydroxylation of either collagen α-chain. Nevertheless, there was a slight tendency towards a higher relative proportion of PYD in highly lysylhydroxylated bone tissue (Fig. 3; $n=36$, $r=0.18$, n.s.).

In Vivo Formed Collagen Fibrils Isolated from Bone and In Vitro Fibrillogenesis—In order to relate the fibril morphology of a total of 10 different human samples to posttranslational modification of the respective specimens,

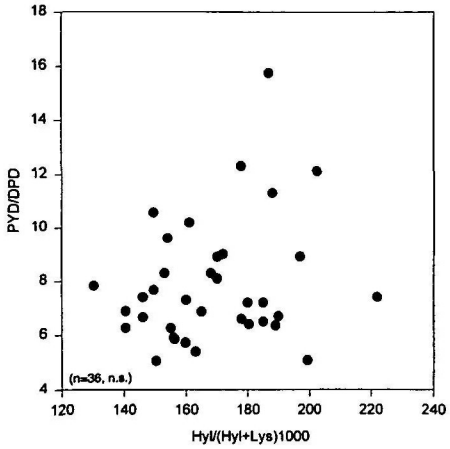


Fig. 3. **Molar ratio of pyridinoline (PYD) and deoxypyridinoline in relation to the degree of lysylhydroxylation of α2(I) [expressed as Hyl/(Hyl+Lys)×1,000].** $n=36$, $r=0.18$, n.s.

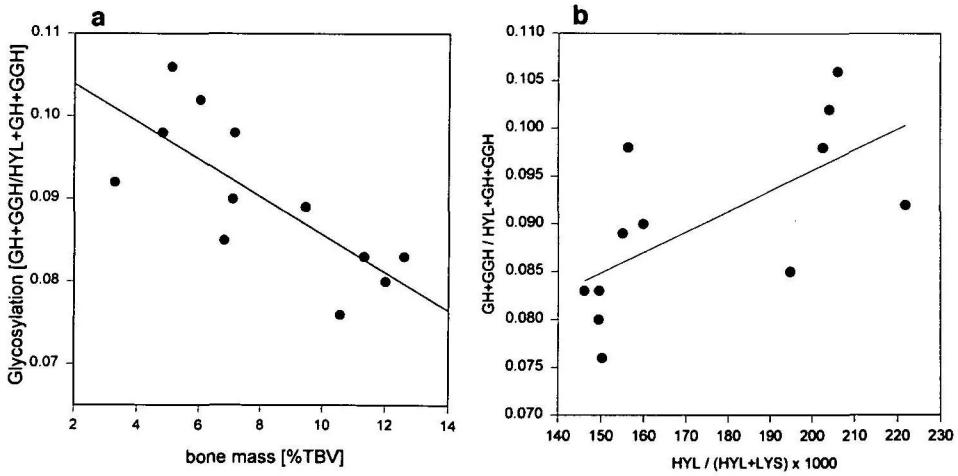
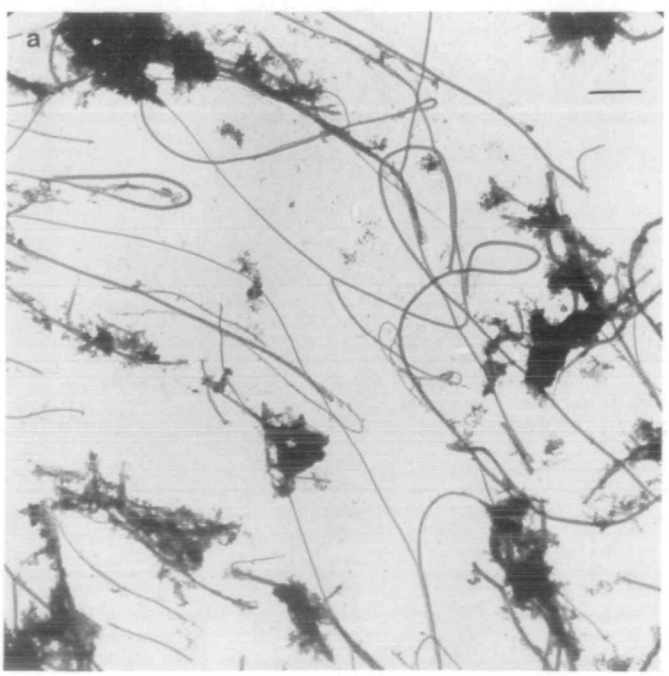


Fig. 2. **a: Negative correlation between the degree of glycosylation of collagen I [expressed as (GH+GGH)/(GH+GGH+Hyl)] and the trabecular bone volume (expressed as % TBV).** $n=12$, $r=-0.75$, $p<0.005$. **b: Positive correlation between the degree of lysyl hydroxylation [expressed as Hyl/(Hyl+Lys)×1,000] and the degree of glycosylation of collagen I [expressed as (GH+GGH)/(GH+GGH+Hyl)].** $n=12$, $r=0.66$, $p<0.01$.



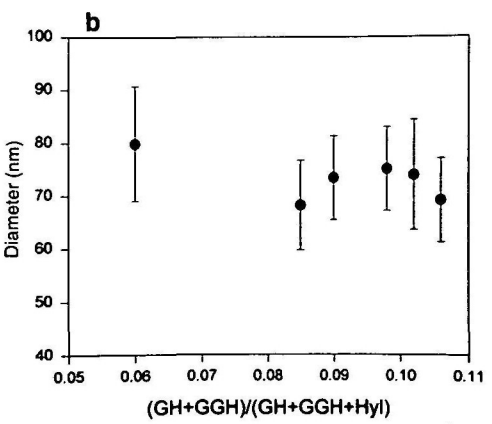


Fig. 4. **a: Representative electron micrograph of collagen I fibrils formed *in vivo* isolated from human vertebral bone (the horizontal bar in the right upper corner represents 1 μm). b: Diameters of collagen I fibrils formed *in vivo* (expressed in nm; mean±standard deviation) in relation to the degree of glycosylation (expressed as GH+GGH/GH+GGH+Hyl).**

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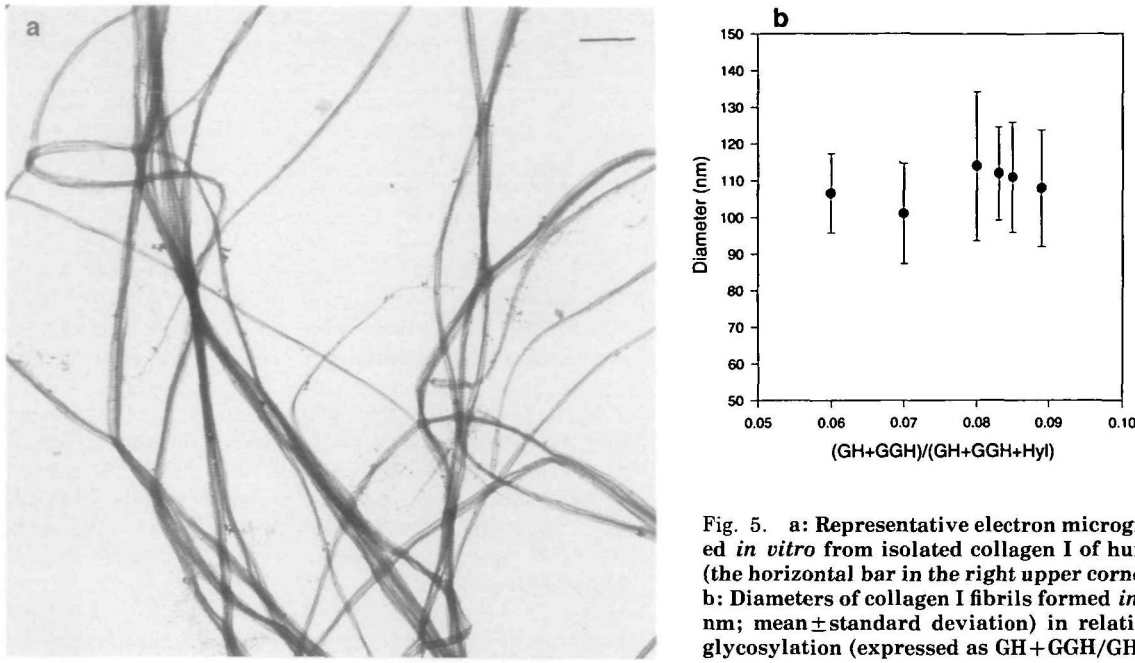

Fig. 5. **a: Representative electron micrograph of fibrils formed *in vitro* from isolated collagen I of human vertebral bone (the horizontal bar in the right upper corner represents 1 μm). b: Diameters of collagen I fibrils formed *in vitro* (expressed in nm; mean ± standard deviation) in relation to the degree of glycosylation (expressed as GH+GGH/GH+GGH+Hyl).**

collagen fibrils were both directly isolated from human bone as well as newly formed *in vitro* from solubilized collagen I. Collagen fibrils (200 per sample) were studied by electron microscopy and analyzed for fibril diameter. A representative electron micrograph, as used for the serial analysis, is shown in Fig. 4a. The results of analysis of *in vivo* formed fibrils are shown in Fig. 4b. There was no clear correlation between the fibril diameter and the degree of glycosylation of collagen I in human vertebral bone.

A representative electron microscopic photograph, as used for the analysis of *in vitro* formed fibrils, is shown in Fig. 5a. The combined results of the *in vitro* fibril formation assays of isolated collagen I are shown in Fig. 5b. Paralleling the results obtained for the fibrils formed *in vivo*, there was no evidence of any relationship between the fibril formation *in vitro* and the degree of glycosylation of collagen I in human vertebral bone.

DISCUSSION

Hydroxylation of lysyl residues is crucially important for the functional integration of individual collagen molecules into the extracellular matrix. Hydroxylysine (Hyl) residues provide the basis for the formation of intermolecular crosslinks and serve as attachment sites for glycoside residues (*7*). While it is the primary function of collagen crosslinks to stabilize the extracellular matrix (ECM) network, the attachment of GH and GGH residues has been hypothesized to play a regulatory role during early steps of ECM formation, *e.g.* fibrillogenesis (*18, 19*). Any change in the extent of lysylhydroxylation (LysOH) may consecutively lead to alterations of the ECM organization.

In the present post-mortem study on vertebral bones of 55 human individuals we found an increased degree of lysylhydroxylation in osteopenic bone of the α1- and α2-chains of collagen I. Since both α-chains are hydroxylated by the same enzyme (lysylhydroxylase) in the same cellular compartment, one would expect similar changes in the degree of lysylhydroxylation of both subunits of collagen I (*7*). The hydroxylysine levels determined in our study represent mean values for the entire pool of solubilized collagen I molecules. Thus we cannot rule out that a mixture of normally hydroxylated and heavily overhydroxylated molecules were liberated from the bone specimens. In osteopenia, the high average level of lysylhydroxylation may reflect repair mechanisms occurring in microfractured bone, since increases in lysylhydroxylation have been observed during bone fracture healing and physiologically in rapidly growing fetal bone (*20-24*). A recent study by Hahn *et al.* revealed that microcallus formation occurs more frequently (1) in females than in males, (2) after the age of 50, and (3) below a TBV threshold value of 11%, whereas a linear correlation between the frequency of microcalli and the TBV was not observed in either normal or osteoporotic spines (*25*). In contrast, our study revealed a significant continuous, negative correlation of lysylhydroxylation with the TBV, only a weak correlation between the lysylhydroxylation of α2(I) and age, and finally only minor differences in lysylhydroxylation between the two sexes. Therefore, it is unlikely that overhydroxylation solely reflects microcallus formation. Another explanation for the high level of lysylhydroxylation is increased bone turnover (*1*). However, high bone turnover does not necessarily occur in osteopenic tissue (*26*) and, to our knowledge, there has been no experimental evidence of increased lysylhydroxylase activity in high turnover, nonfractured bone tissue. Overall, microcallus formation and/or increased bone turnover are unlikely to represent major contributors to the high level of lysylhydroxylation observed in osteopenia. Our recent finding of an inhibitory effect of TGF-β on lysylhydroxylation *via* pretranslational control of lysylhydroxylase activity rather suggests a local regulatory imbalance, *e.g.* alteration in growth factor activity and/or responsiveness as a possible cause for the qualitative changes of the collagenous bone matrix in osteopenia (*8*).

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In bone, PYD and DPD are important mature, nonreducible crosslinks. While the synthesis of PYD involves three Hyl residues, the formation of DPD requires 2 Hyl and one lysine residue (*27*). Consequently, any change in the degree of LysOH and thus in the availability of Hyl residues may affect the molar ratio of PYD and DPD, as observed, for example, in Ehlers-Danlos-Syndrome VI, where the relative proportion of PYD is low (*28, 29*). An elevated PYD/DPD ratio has recently been reported in overhydroxylated, osteoporotic avian bone but not in overhydroxylated bone from patients with osteogenesis imperfecta (*1, 30*). Our analysis of human vertebral bone showed a weak trend similar to that observed in avian bone, without, in contrast, being significant, which may reflect species differences. However, the changes in LysOH (ranging from 14.5 to 22.5% hydroxylated lysine residues) associated with alteration of the bone mass were lower than those observed in avian bone (*1*). While we could not measure the degree of lysylhydroxylation of the telopeptides—which may be catalyzed by a different lysylhydroxylase (*31*)—in the pepsinized material, the determination of crosslink components was performed on non-pepsinized bone powder. Thus we conclude that the observed changes in lysylhydroxylation within the tripelhelical part of the collagen molecule are probably too small to have a major influence on either the tissue concentrations or the molar ratio of the two crosslink components. Based on our data, it appears unlikely that an altered rate of formation of either crosslink component is responsible for the biomechanical instability recently reported for overhydroxylated murine and avian bone tissue (*1, 2*). However, at present it can not be excluded that changes in the immature, reducible crosslinks may play a role (*6*).

Like crosslink formation, the enzymatic glycosylation of collagen molecules also involves Hyl residues, and the glycosylated residues probably fulfill distinct, yet unknown functional demands. We observed a highly significant increase in collagen glycosylation in collagen I of osteopenic bone. This was most likely a consequence of increased lysylhydroxylation, since there was also a significant positive correlation between glycosylation and hydroxylation. Therefore, our data suggest that the degree of lysylhydroxylation, as observed in pepsin-digested collagen, is closely related to the glycosylation level, while the modification of the helical part of the collagen molecule seems not to have an influence on the relative proportions of the two mature crosslinks investigated.

The precise functions of GH and GGH residues are still not fully known, although they are likely to modify physicochemical properties, such as hydrophobicity and susceptibility to proteolysis (*7*). Since the increased numbers of GH and GGH residues reduce the hydrophobicity necessary for collagen aggregation, the fibril formation may also be influenced by glycoside residues (*19*). There is recent experimental evidence that even small increases in glycosylation in a similar range to that observed in the present study influence several steps in fibril formation and reduce the diameter of newly formed fibrils of collagen (*32, 19*). It is fair to assume, therefore, that lysyl overhydroxylation, as observed in osteopenia, may alter the fibrillar structure of the collagenous bone matrix *via* an increased degree of glycosylation. Accordingly, Bailey *et al.* reported smaller fibrils in osteoporotic femoral heads compared to in controls (*33*). In our investigation on a total of 10 different human samples, both the results of analysis of the *in vivo* formed collagen I fibrils as well as those of the *in vitro* fibrillogenesis experiments with extracted human bone collagen I argue against an influence of enzymatic glycosylation on the diameter of fibrils, at least in the range observed. Although a direct comparison of fibrils derived from pepsin-treated, purified collagen lacking non-helical sites and fibrils formed *in vivo* from intact collagen is not possible, the results obtained in two methodologically different experiments suggest a similar interpretation. The different diameters observed in the two assays might be due to that the fibrils isolated from bone are composed of collagens I and V; the latter have been reported to reduce the diameter of collagen I fibrils in a concentration-dependent manner (*34*). Although in general collagen molecules with a higher degree of glycosylation form smaller fibrils (*18, 34*), the differences observed here are possibly still within the range of naturally occurring variation of collagen fibril diameters and too small to be distinguished with statistical significance.

In conclusion, a low trabecular bone volume of human vertebrae is negatively correlated with the degree of lysylhydroxylation of both α-chains of collagen I. These changes are paralleled by a higher level of glycosylation, the latter also being significantly, and negatively correlated with the TBV. In human vertebral bone, an increased level of lysylhydroxylation does not have a significant influence on the tissue concentrations or the molar ratio of PYD and DPD. Furthermore, an increased glycosylation level of collagen I is not associated with the formation of fibrils of smaller diameters. Future studies will have to elucidate the impact of collagen modification on the formation of collagen fibers, immature crosslinks and the recently identified pyrrole crosslink in order to clarify the role of an altered collagen modification in the functional integrity of the collagenous bone matrix.

We would like to thank Mrs. C. Sesselmann and Mrs. K. Wießmann for their expert technical assistance. We are also grateful for the cooperation of the Department of Pathology of the Medizinische Universität zu Lübeck regarding the vertebral bone samples.

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