Purification and Partial Characterization of Small Proteoglycans I and II, Bone Sialoproteins I and II, and Osteonectin from the Mineral Compartment of Developing Human Bone*

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Using nondegradative isolation procedures we purified and characterized five major noncollagenous proteins from developing human bone. Small bone proteoglycan I, M, ~ 350,000 on sodium dodecyl sulfate (SDS), 4-20% gradient polyacrylamide gels has a different amino-terminal sequence of NH2-Asp-Glu-Glu-( )-Gly-Ala-Asp-Thr and is not cross-reactive with the small bone proteoglycan II, M, ~ 200,000 on SDS-gradient polyacrylamide gels. Bone proteoglycan II is 95% N terminally blocked and the small amount that can be sequenced has an amino-terminal sequence (NH2-Asp-Glu-Ala-( )-Gly-Ile.. ) that is apparently similar but not identical to a small proteoglycan isolated by Brennan, M. J., Oldberg, A., Pierschbacher, M. D., and Ruoslahti, E. (1984) J. Biol. Chem. 259, 13742-13750 from human fetal placenta membrane. Two bone sialoproteins, each of which migrates at a M, ~ 80,000 on SDS gels, have also been isolated. Bone sialoprotein I has an amino-terminal sequence of NH2-Ile-Pro-Val-Lys-Gln-Ala... which is different from that of bone sialoprotein II with an amino-terminal sequence of NH2-Phe-Ser-Met-Lys-Asn-Lca... The two bone sialoproteins do not cross-react on Western blot analysis. Human bone osteonectin contains a large number of cysteines, more than 90% of which appear to be in disulfide bonds. The N-terminal amino acid sequence of human bone osteonectin was nearly identical to bovine bone osteonectin and had many similarities to a protein found in mouse parietal endoderm (Mason, I. J., Taylor, A., Williams, J. G., Sage, H., and Hogan, B. L. M. (1986) EMBO J. 5, 1831-1837).

Noncollagenous proteins constitute about 10% of the organic matrix of mammalian bone. While no noncollagenous protein has had its biological role unambiguously assigned, it is generally agreed that the secretion, assembly, maturation, mineralization, and maintenance of the bone collagen matrix may be aided or directed by one or more of these proteins (for review see Refs. 1-3). Several proteins in the mineral compartment, including serum albumin (4) and the αHS glycoprotein (5) are serum components that bind to the bone hydroxyapatite crystals. Other bone proteins are synthesized by bone-derived cells in culture, including bone proteoglycans I and II (6, 7), bone sialoprotein II (6), osteonectin (7), and osteocalcin, also known as bone gla protein (8).

The mineral compartment of developing bovine bone has been shown to contain two small chondroitin sulfate proteoglycans as distinguished by SDS-polyacrylamide gel electrophoresis (9). The two bovine bone proteoglycans differed in amino acid composition (proteoglycan I being more rich in leucine) and in apparent lack of cross-reactivity of proteoglycan I with antisera made to proteoglycan II (10). It was suggested that proteoglycan I contains two chondroitin sulfate chains and proteoglycan II, one chain (10). Recent work with limited V-8 protease digestion of proteoglycan II-like molecules from bovine bone, cartilage, and tendon suggests that while these molecules are closely related, the bone proteoglycan II core protein is subtly different from those in the other connective tissues (11).

During the mid 1960s Herring and co-workers (12) purified and characterized a sialic acid-rich protein of approximately 25,000 daltons from bovine bone. This 25,000 M, bovine bone sialoprotein has recently been shown to be a degradation product of a larger molecule, bone sialoprotein II (13, 14). This intact glycoprotein is 70,000-80,000 M, on SDS gels and contains approximately 50% protein, 12% sialic acid, 7% glucosamine, and 6% galactosamine. Bovine bone sialoprotein II does not stain with Coomassie unless pretreated with neuraminidase (13) but can be easily stained with Alcin blue or Stains All. A second bone glycoprotein, sialoprotein I, has been identified in bovine bone (2, 14).

Osteonectin, a phosphorylated glycoprotein with an apparent molecular weight of 38,000 on SDS gels (15, 16), is a dominant (10-15%) noncollagenous protein in the mineral compartment of developing bovine bone (17). Other fetal bovine tissues have been shown by radioimmune assay to contain small quantities of a cross-reactive species, although the levels are only 0.1-0.2% of that found in bone (17). mRNA for osteonectin has also been shown to be present in tendon tissue and in a variety of cultured cells (18). Osteonectin-like products have been shown to be present in serum (17), at least some of which appears to be associated with platelets (19). The function of osteonectin in bone is unknown but it has been shown to initiate the formation of hydroxyapatite onto collagen in vitro (20). Osteonectin binds to collagen fibrils, calcium, and hydroxyapatite, the last with high affinity (16, 20). Further, the levels of bone osteonectin in one bovine model of osteogenesis imperfecta (Texas variant) was severely depressed compared to the bone of unaffected siblings (21). Affected individuals of a second bovine model of osteogenesis imperfecta (Australia variant) have clinically identical symptoms to the first model but have normal levels of bone osteonectin (22), suggesting that bone osteonectin content is a biochemical marker that may be useful in distinguishing.

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Bone proteoglycans are distinctly different from each other as chromatographed on a freshly prepared DEAE-Sephacel (Pharmacia) column. The bone proteoglycans were a generous gift of Drs. G. Tschank and H. M. Hanauske-Abel of the Prenatal Diagnosis Laboratory, Gutenberg University Mainz, West Germany and Dr. Stuart Weinstein, University of Iowa. To prepare each, 30 g of milled bone was extracted in 4 l of 0.1 M guanidine HCl, (Bethesda Research Laboratories (BRL)) 0.05 M Tris (BRL), 0.1 M 6-aminooacaproic acid (Sigma), 5 mM benzamidine HCl (Behring Diagnostics), and 1 mM phenylmethylsulfonyl fluoride (BRL), pH 7.4, for 48-72 h at 4 °C as described previously (23). The supernatant was poured off, the insoluble material rinsed twice with 100 ml of fresh buffer, and then stirred for 72 h at 4 °C in a demineralizing buffer that contained all of the above plus 0.5 M tetrasodium EDTA (Sigma). The supernatant was clarified by filtering through Whatman No. 4 paper and then concentrated to a volume of 60 ml by ultrafiltration (Amicon YM-10 membranes). One-third of each preparation was filtered (0.45 μm filter) and chromatographed on tandem Sepharose CL-6B (Pharmacia) columns (2.6 × 190 cm) in 4 M guanidine HCl, 0.05 M Tris, pH 7.4, as described previously (15), except that the flow rate was 18 ml/h. The elution profile was monitored at 234 nm, and 19-min fractions were collected. Appropriate fractions from the Sepharose CL-6B columns were pooled, concentrated by ultrafiltration, and the buffer exchanged to fresh 7 M urea, 0.05 M Tris, pH 6.0, by repeated concentration and dilution in the Amicon-stirred cell at 4 °C. The sample was then chromatographed on a freshly prepared DEAE-Sephacel (Pharmacia) column (1.6 × 6 cm) equilibrated in the urea buffer. The sample was loaded and the column washed at 30 ml/h until the base line (226-240 nm) was re-established. A linear 16-h gradient from 0 to 1 M NaCl in the same buffer was used to elute the proteins, and 10-min fractions were collected. All samples in urea were either concentrated by ultrafiltration and rapidly desalted (see below) or stored at -20 °C. Individual fractions from either the Sepharose CL-6B or the DEAE-Sephacel column were often monitored by SDS-polyacrylamide gel electrophoresis (see below) by centrifuging 0.2-1.0 ml of a fraction in a Beckman 10 or 30 microcentrifugation cell (Amicon) and flushing with excess water. The sample was recovered from the membrane using electrophoresis sample buffer containing an excess of sucrose and electrophoresed as described below.

Protein Preparation—For each preparation, 30 g of milled bone was extracted in 4 l of 0.1 M guanidine HCl, 0.05 M Tris, pH 7.4, NaCl in the same buffer was used to elute the proteins, and 10-min fractions were collected. All samples in urea were either concentrated by ultrafiltration and rapidly desalted (see below) or stored at -20 °C. Individual fractions from either the Sepharose CL-6B or the DEAE-Sephacel column were often monitored by SDS-polyacrylamide gel electrophoresis (see below) by centrifuging 0.2-1.0 ml of a fraction in a Beckman 10 or 30 microcentrifugation cell (Amicon) and flushing with excess water. The sample was recovered from the membrane using electrophoresis sample buffer containing an excess of sucrose and electrophoresed as described below.

Desalting—Due to the presence of endogenous proteases that appear to be active during dialysis (24) all samples (up to 50 ml) were rapidly desalted by chromatography on Trisacryl GF 05 (LKB). The column (2.6 × 34 cm) was run at 90 ml/h in 0.1 M ammonium acetate and monitored at either 226 or 280 nm. Proteins (>2500 M,) eluted in the excluded volume were immediately frozen and lyophilized. Because we noted that most commercial sources of ammonium acetate contain significant levels of glycine, samples used for amino acid analysis were desalted in 0.1 M acetic acid brought to a pH of 7.0 with ammonium hydroxide.

Enzymatic Digestion—50-500 μg dry weight of each bone proteoglycan I or II were digested with 10 milliliters of chondroitinase ABC (Miles) in 25 μl of 0.05 M Tris, 0.063 M NaCl, 0.25 mg/ml bovine serum albumin for 1 h at 37 °C (9). The digestion was stopped by boiling for 2 min in an equal volume of SDS sample buffer.

Polyacrylamide Gel Electrophoresis and Electrot blotting—Polyacrylamide gels (4-20%) SDS slab gels (16) × 140 × 1.5 mm were then formed and topped with a 3% stacking gel as described previously (9). In addition to the Coomassie and Alcian blue staining (9), Stains All was used (23). Electrotransfer of proteins out of the SDS gels and onto nitrocellulose sheets (Schleicher & Schuell) was accomplished in 15-60 min at 100 V in a Bio-Rad Trans blot apparatus with cooling to 4 °C according to the method of Towbin et al. (25).

Analytical Procedures—Amino acid compositions were determined on samples hydrolized in 6 N HCl in vials sealed under nitrogen for 20 h at 110 °C. Analyses were obtained on a single cation-exchange column as described elsewhere (26). Detection of the amino acids was by ninhydrin complex monitored at 570 and 440 nm. Phosphoserine content was determined by a modification of the method of Cohensol et al. (27). Sodium citrate buffer (0.15 N, pH 1.8) was used to give base-line separation of phosphoserine and phosphothreonine. Detection was by o-phthalaldehyde fluorescence (28).

Sialic acid were determined by the method of Jourdain et al. (29). Automated Protein Sequencing—Lyophilized samples were dissolved in 1% trifluoroacetic acid and subjected to automated Edman degradations using an Applied Biosystems model 470A gas-phase sequencer employing the standard "NoVac" program supplied by the manufacturer (30). Phenylthiohydantoin derivatives were identified by HPLC on an IBM cyano column (31). The HPLC system used with this column consisted of a Perkin-Elmer series 4 liquid chromatograph, an LC-85B spectrophotometric detector equipped with a 1.4-μl flow cell, an LCI 100 computing integrator, and a model 7500 computer employing Chrom III software. These analyses were supplied under contract by the University of California at San Diego.

Immunodetection—Nitrocellulose electrotransfers were processed for immunodetection as described previously (13) with the following changes: (a) 0.05% Tween 20 (Sigma) was present in all but the last wash immediately prior to the addition of the 4-chloro-1-naphthol reagent, and (b) normal goat serum (GIBCO) was present at a dilution of 1:500 in the last half of the bovine serum albumin-blocking procedure and in the first and second antibody incubations. Peroxidase-conjugated goat anti(rabbit) IgG (1:1000) was purchased from Kirkegaard and Perry Laboratories, Inc.

Antisera Production—Rabbits were injected at multiple sites both intradermally and intramuscularly with an emulsion of protein in phosphate-buffered saline and either Freund’s complete adjuvant (1st...
FIG. 2. Elution profile of the total mineral compartment extract on Sepharose CL-6B under denaturing conditions (4 M guanidine HCl, GdmCl) (top) and SDS gel of representative fractions (bottom). Bars indicate the range over which individual proteins can be found in significant amounts. Elution conditions are detailed under "Experimental Procedures." One ml each of representative pooled fractions was concentrated and desalted by ultrafiltration (Centricon 30 for fractions 1-7 and Centricon 10 for fraction 8), recovered from the membrane in SDS sample buffer, electrophoresed on a 4-20% gradient polyacrylamide gel (reduced), and stained with Coomassie Blue and Alcian blue. Human serum albumin (hsa), procollagen (procol), and osteocalcin or bone gla protein (oc). Other abbreviations as in Fig. 1.

FIG. 3. Elution profile of the mineral compartment proteins (fractions 85-125 from Sepharose CL-6B) on DEAE-Sepharose cel under denaturing conditions (top) and SDS gel of representative fractions (bottom). This unusually broad range of molecular weight fractions was taken from the Sepharose CL-6B column to illustrate the elution positions of the various proteins. Open squares show the elution gradient. Braces show location of the proteins as identified by SDS-gel electrophoresis. Representative fractions were desalted (Centricon 30) and electrophoresed on 4-20% gradient polyacrylamide gel (SDS, reduced) and stained with Coomassie Blue and Alcian blue. Abbreviations are as in Fig. 1.

RESULTS AND DISCUSSION
We have shown earlier that developing bone is a good source of mineral compartment proteins (most of the noncollagenous proteins are degraded in older bone (24)). The first denaturing extract is used to remove the proteins associated with the soft tissues of the bone, including cells, blood, and adhering connective tissues (32). The second, demineralizing extract contains virtually all of the mineral compartment noncollagenous...
osteonectin

In, on this column.

denaturing conditions.

Open squares

electrophoresis and Western blot/immunodetection.

weight standards.

dient polyacrylamide gels and stained with Alcian blue and Coomassie

Blue

Coomassie Blue

proteins of the bone, including serum-derived products (such

as serum albumin and oHS glycoprotein) that are absorbed

to the crystal surfaces as well as proteins thought to be an

integral part of the matrix. Fig. 1 shows a SDS gel of the toul

complement of proteins in the mineral compartment of de-

veloping human bone. Several proteins are high in relative

abundance and are described in this report. Approximately

95% of the glycosaminoglycan contained in the mineral com-

partment of bone is in the two small proteoglycans I and II

(9). Proteoglycan I is thought to contain two chondroitin

sulfate chains (M, ~ 40,000 each) attached to a core protein

of M, ~ 45,000 (10). Proteoglycan II, a faster migrating species

on SDS gels, is thought to contain only one chondroitin

sulfate chain on a similar-sized protein core (10). Differences

in the amino acid compositions of these two proteoglycans

from bovine bone and a potential difference in immunogenic-

ity and cross-reactivity had been noted (10).

To purify proteoglycans I and II, we relied on the separation

of the two proteoglycans on Sepharose CL-6B under denatur-

ing conditions, using SDS gels to analyze individual fractions

from the column. We pooled high molecular weight fractions

(the leading half of the bar labeled proteoglycan I in the top

panel of Fig. 2, typically fractions 67–75) in the isolation of

proteoglycan I, and low molecular weight fractions (the trail-

ing half of the bar labeled proteoglycan II on Fig. 2, typically

fractions 82–89) for proteoglycan II. The bottom panel of Fig.

2 shows representative fractions of each major peak areas

electrophoresed on SDS-polyacrylamide gels. The peak

fractions also correspond to the peak numbers first described for

the bovine bone noncollagenous proteins described earlier

(15). DEAE-Sepharose chromatography, while not useful in

separating bone proteoglycan I from bone proteoglycan II was

used in each case to remove nonproteoglycan proteins from

the pooled fractions (see top panel, Fig. 3). The bottom panel

of Fig. 3 shows representative fractions of the DEAE column,

desalted, and electrophoresed on an SDS-polyacrylamide gel.

Final purification was accomplished with the MONO Q anion-

exchange column (Fig. 4).

Both the final intact products and their core proteins (gen-

erated by digestion with chondroitinase ABC to remove the

attached chondroitin sulfate chains) are shown in Fig. 5. The

selection of the fractions for the larger of the proteoglycan I

and the smaller proteoglycan II molecules to overcome the

propensity of the two heterogeneous populations to co-elute to
TABLE II  

Amino acid sequences  

|                | 1  | 6  | 11 | 16 | 21 | 26 | 31 | 36 |
|----------------|----|----|----|----|----|----|----|----|
| Human bone proteoglycan I | NH₂ DE(E)AX | GADT(S) | GVLDP | DSVT | TYSAM | XPF | G   |
| Human bone proteoglycan II | NH₂ D(E)AXG | I(I)PEV | PDD(R) (P) | FEP  |
| Human fetal membrane PG* | NH₂ DEAXG | IGPEY | PDDRD | FEP  |
| Human bone sialoprotein II | NH₂ IPVKQ | ADSEG | XEQXQ | LYNN(Y) |
| Human bone sialoprotein | NH₂ FSMKN | LHRV | KIERS | EE(N)GV | FKYRP | XY(Y) | LX | KXAYF | X(P)X |
| Human bone osteonectin | NH₂ APQQE | ALPDE | TEVVE | ETVAE | VTEVP | TGANP | VQVES | G   |
| Bovine bone osteonectin* | NH₂ APQXE | ALPDE | XEVVE | XEVAE | VAEPV | VGANP | VXXEV | G   |
| Mouse SPARC* | NH₂ APQQT | EVAEE | I-VEE | ETVVE | ETGPV | VGANP | VQVEM | G   |

* Ref. 33.  
* Ref. 18.  
* Ref. 35.  

**FIG. 6.** Detection of purified bone sialoprotein I and II by SDS-gel electrophoresis (a) and Western blots followed by immunodetection using antisera against bone sialoprotein I (b) and bone sialoprotein II (c). 10 μg of each protein was electrophoresed on the SDS 4–20% gradient polyacrylamide gels and stained with Stains All (a). A similar gel was used for electrophoretting the proteins and detection by antisera directed against bone sialoprotein I (b) or II (c). Immunodetection by peroxidase-conjugated second antibody and 4-chloro-1-naphthol is described under “Experimental Procedures.”  

some degree did, however, skew the apparent molecular weight of the purified proteoglycans (thus purified proteoglycan I was apparently larger than the average proteoglycan I and the purified proteoglycan II population apparently smaller). For this reason we have chosen to assign the $M_r$ of each proteoglycan to the original crude extract as seen in Fig. 1 to more accurately describe the average size of the proteoglycans. Therefore, the estimated $M_r$ of proteoglycans I and II are 350,000 and 200,000, respectively, although the $M_r$ of any small proteoglycan is, as always, a function of the SDS gel system used. The lanes in Fig. 5a are overloaded to show the relative purity of proteoglycan I and proteoglycan II preparations. The minor components seen at lower molecular weight in the proteoglycan II lane may be some free chondroitin sulfate chains or some minor amounts of proteoglycan II breakdown products, but they did not interfere with the N-terminal amino acid sequence described below.
glycan I molecule (Table II) are similar to that of proteoglycan II (with an extra glutamic acid at position 3), suggesting that position 5 of proteoglycan I may serve as a potential site for the addition of a glycosaminoglycan chain. No direct evidence of a substituted serine is yet available, however. Interestingly, a second serine-glycine sequence is seen at positions 10 and 11. In sequence analysis, the signal for the position 10 serine was relatively weak, indicating that a glycosaminoglycan may be present at this location also.

The two bone sialoproteins in the mineral compartment of human bone are very similar to those found in bovine bone (2, 13, 14). Bone sialoprotein I, so named because it elutes at a lower salt concentration from ion-exchange columns, is similar in size to bone sialoprotein II on SDS-polyacrylamide electrophoretic gels but chromatographed as a slightly smaller protein on Sepharose CL-6B under denaturing conditions (Fig. 2). To purify these two proteins, pooled fractions from the molecular sieve column (bars, Fig. 2) were concentrated and exchanged into urea buffer by ultrafiltration and chromatographed first on DEAE-Sephacel, then on MONO Q (see “Experimental Procedures” and Figs. 3 and 4). Unlike bovine bone sialoprotein I which stained poorly with Coomassie Blue and not at all with Alcian blue, human bone sialoprotein I did not stain well with either dye. Stains All, however, gave a strong blue-purple reaction product (typical of highly acidic proteins) not stain well with either dye. Stains All, however, gave a strong blue-purple reaction product (typical of highly acidic proteins) for both human bone sialoprotein I (Fig. 6a) and its bovine counterpart (data not shown). The N-terminal sequence of human bone sialoprotein I shown in Table II had several cycles with no identifiable amino acid residues. Both oligosaccharide attachment sites and cysteine residues lead to a lack of an identifiable amino acid in sequence analysis.

The purified human bone sialoprotein I, like its bovine counterpart (13), did not stain with Coomassie Blue under our routine conditions but did stain with Alcian blue (lane 4, bottom panel of Fig. 3). Fig. 6a shows this protein on SDS-polyacrylamide gel electrophoresis, stained with Stains All. Antisera made against bone sialoprotein I did not cross-react with bone sialoprotein II or vice versa on Western blots (Fig. 6, b and c) indicating that these proteins are not closely related. This is in contrast to partial cross-reactivity reported by Franzen and Heinegard (14) for the two bovine sialoproteins. Further evidence that the two sialoproteins are different are their different amino acid compositions (Table I) and different N-terminal sequences (Table II). As was the case for bone sialoprotein I, the sequence of bone sialoprotein II has several analytical cycles with no identifiable amino acids, indicating either post-translational modification sites or cysteine residues.

Purified human osteonectin, like its bovine counterpart (15, 16) is a phosphorylated glycoprotein of Mr, ~46,000 on reduced SDS gels (Fig. 7). Unreduced, the protein is considerably smaller on SDS gels (Mr, ~38,000, Fig. 7) suggesting that it contains several intramolecular disulfide bonds. This large change in Mr upon reduction was reported earlier for fetal porcine osteonectin (34). Both human and bovine bone osteonectin incorporated much more of the radiolabeled alkylating agent, [14C]iodoacetamide, after reduction with dithiothreitol (13.9- and 15.4-fold increases, respectively) compared with the same protein unreduced. This suggests that most (>90%) of the cysteines in the bone osteonectin are incorporated into disulfide bonds. Table II shows that the N-terminal sequences of bovine and human bone osteonectin are highly conserved, indeed there are only two conserved changes (positions 22 and 26). In contrast, a sequence for a related protein from mouse parietal endoderm (derived from cDNA, Ref. 35) contains many changes in the N terminus. It is an open question at this time whether the differences between the human/bovine bone-derived protein and the soft tissue mouse protein arise from species differences (in a nonconserved region) or from a tissue-specific difference within a given species. The latter possibly has at least two precedents in fibronectin (36) and α-crystallin (37) in which a single gene results in two closely related but different primary structure proteins.

Table III shows the cross-reactivity of the various antisera produced against four of the five human bone-derived proteins described in this report to a wide variety of species. For each species, the bones were cleaned, milled under liquid nitrogen, extracted first with the 4 M guanidine buffer, then with the demineralizing buffer. Lyophilized mineral compartment extract (200 µg) was electrophoresed on our standard SDS gel, electrotransferred for 20 min, and detected using the appropriate antiserum and peroxidase-conjugated second antiserum.

In this report we have presented evidence that the two small proteoglycans of developing human bone (I and II) are different proteins. We also have shown that the two human bone sialoproteins, I and II, are different proteins. Human bone osteonectin contains a substantial number of disulfide bonds and has an N terminus that is highly conserved with respect to bovine bone osteonectin. Using the standard nomenclature recommended by the American Society of Bone and Mineral Research (in which each protein, or core protein in the case of proteoglycans, is named by its first three amino-terminal amino acids and its apparent molecular weight on a 4–20% gradient polyacrylamide SDS, reduced gel), the bone proteins described in this report are identified as: DEE-46,000 (proteoglycan I), DEA-47,000 (proteoglycan II), IPV-80,000 (bone sialoprotein I), FSM-80,000 (bone sialoprotein II), and APQ-46,000 (osteonectin).

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