# Thrombospondin Is an Osteoblast-derived Component of Mineralized Extracellular Matrix

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*Abstract.* Thrombospondin, the most abundant protein of platelet α granules, is a biosynthetic product of a variety of connective tissue cells and a component of many extracellular matrices. In this study, thrombospondin distribution in bone was investigated using a monoclonal antibody specific for the human protein. Thrombospondin was localized in osteoid of undemineralized, frozen sections of fetal subperiosteal bone, and identified as a component of mineralized bone matrix of neonatal and/or young (growing) bone of many animal species by Western blot analysis. Adult human bone cells were demonstrated to contain mRNA for thrombospondin by hybridization of a cDNA thrombospondin probe to a 6.1 kb mRNA. Pulse–chase experiments indicated that the protein was synthesized and the majority was secreted from osteoblastic cells. Treatment of the cells with TGF-β (0.01–10 ng/ml) slightly decreased total thrombospondin synthesis, but caused an increase in the retention on newly synthesized thrombospondin in the cell layer/matrix fraction. In cell attachment assays, thrombospondin mediated adhesion, but not spreading of adult human bone cells.

THROMBOSPONDIN, a trimeric molecule with $M_r = 450,000$, is composed of inter- and intramolecular disulfide linked monomeric subunits of ∼150 kD, with a minor globular domain at the amino terminus connected by an intervening stalk to a major globular region at the carboxyl terminus. Distinct binding regions have been identified, and analysis of the amino acid sequence determined from cDNA clones (15, 31, 35) has revealed many sequence homologies with proteins associated with clot formation, and with connective tissue matrices (reviewed in 20, 34, 40, 59). The amino terminus contains a heparin binding region (13, 36, 47) with a sequence similar to fibrinogen (15). The stalk region contains a collagen binding site (33, 47) and a cysteine-rich region involved in disulfide bonding (21), and shares sequence homology to the precursor amino terminal extensions of the α1 chain of types I and III collagens and to von Willebrand factor (27). The stalk region also contains binding sites for thrombin (11), fibrinogen (4, 12, 47), fibronectin (33, 47), laminin (47), plasminogen (47, 57), and plasminogen activator (58). In addition, this region contains sequence homologies with circumsporozoite protein from *Plasmodium falciparum* and EGF (35). The carboxy globular domain contains a Ca⁺⁺ binding region homologous to calmodulin, paralbumin, and fibrinogen (36), but due to several sequence differences, the molecular conformation is not comparable to the E-F hand structure identified in calmodulin. This region also contains RGD (35), a sequence that has been implicated in cell attachment, and indeed, thrombospondin appears to mediate cell attachment in a variety of cell types (53, 64, 65). However, it is not clear what role the RGD sequence plays in thrombospondin-mediated cell attachment. The most carboxy terminal portion of this domain appears to be involved in mediating platelet aggregation (3, 14, 38) and binds to the histidine-rich glycoprotein of serum (39).

Although first identified as a major protein of human platelet α granules (2) and presumably a product of the megakaryocyte, thrombospondin is widely distributed in tissues (69), and is also produced by a variety of cell types including endothelial cells (35, 45, 46, 52), fibroblasts (28), monocytes and macrophages (29), aortic smooth muscle cells (52), granular type II pneumocytes (55), keratinocytes (65, 68), and glial cells (1). In this study, thrombospondin was identified as a biosynthetic product of bone cells that becomes incorporated into the mineralized matrix of bone.

## Materials and Methods

### Indirect Immunofluorescence

Identification of thrombospondin in the osteoid layer of developing bone was determined by indirect immunofluorescence as described previously (67). Undemineralized, frozen sections (10 μ thickness) of subperiosteal fetal bovine (5 mo gestation) and fetal human (∼4 mo gestation) bone were incubated with a 1:20 dilution of monoclonal mouse anti–human platelet thrombospondin ascites fluid containing ∼7 mg/ml IgG (kindly provided by Dr. Deane F. Mosher, University of Wisconsin), and subsequently with a

1:16 dilution of fluorescein-labeled rabbit anti–mouse IgG (0.5 mg affinity-purified IgG per milliliter) (Organon Teknika-Cappel, Malvern, PA). For comparative purposes, similar sections were reacted with monoclonal mouse anti–bovine osteonectin (6) (~6 mg/ml IgG per milliliter of ascites fluid) and normal mouse serum (1:10 dilution, control). Sections were examined with a Leitz ultraviolet microscope equipped with a 35-mm camera. Photomicrographs of antibody and normal mouse serum–treated sections were exposed and processed under identical conditions.

## Western Blot Analysis of Mineralized Matrix Extracts

Bone from a variety of animal species was processed as described in detail elsewhere (62). Samples were initially extracted with buffered 4 M guanidine hydrochloride containing protease inhibitors ("G" extract) to remove cellular components and proteins not intimately associated with mineral and subsequently with the same solvent but containing 0.5 M EDTA ("E" extract) to extract proteins bound to hydroxyapatite. The mineralized matrix proteins were subjected to electrophoresis on 12% polyacrylamide gels (32) with minor modifications (18) and electrotransferred on to nitrocellulose (63). Immunodetection of thrombospondin in these Western blots was performed as described previously (63) with minor modifications (30) using a 1:2,000 dilution of monoclonal mouse anti–human thrombospondin and a 1:2,000 dilution of peroxidase-conjugated rabbit anti–mouse IgG.

## Bone Cell Culture

Cultures of fetal (generously provided by Dr. Jeffrey B. Kopp, National Institute of Dental Research, Bethesda, MD) and adult human bone cells were established as previously described with minor modifications (23). Femoral subperiosteal scrapings (from prenatal material >14 wk gestational age) or trabecular bone (generally from femoral heads removed during hip replacement) were minced into very small fragments (~1 × 1 mm) and washed extensively with serum-free nutrient medium to remove blood and soft connective tissue. The washed fragments were then treated with 250 U/ml bacterial collagenase (Type IV; Sigma Chemical Co., St. Louis, MO) for 2 h at 37°C with constant rotation. The fragments were washed extensively with serum-free medium to remove cells released by the collagenase. The treated fragments were placed in calcium-free DME (Biofluids, Inc., Rockville, MD) containing 4.5 g glucose/liter, 10% heat-inactivated FBS (Gibco Laboratories, Grand Island, NY), 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 50 μg/ml ascorbic acid. Due to the presence of FBS, the final calcium concentration was ~0.2 mM. Cultures were maintained in a humidified atmosphere of 90% air, 10% $CO_2$ (to obtain appropriate pH levels in DME) and the medium was replaced every 2–3 d. After ~2 wk, cells emerged from the chips, and after another 1–2 mo, the cultures became confluent. The cells were passaged by release with Typsin–EDTA (Gibco Laboratories) and plated at specific densities as noted below in DME replete with calcium and containing the same additives as noted above ("complete" medium). Previous studies have indicated that these cultures are osteoblastic in nature based on their high levels of alkaline phosphatase activity, cAMP formation in response to parathyroid hormone, and the biosynthesis of an extracellular matrix containing the appropriate proteins (23).

## Isolation of mRNA and Northern Blot Analysis

mRNA was isolated from the adult human bone cell cultures, as well as from cultures of human skin fibroblasts, periodontal ligament cells, and gingival cells as previously described (8, 71). In addition, mRNA was also isolated from human decidual tissue (66). Equal amounts of isolated mRNA (10 μg) were subjected to electrophoresis in 1.2% formaldehyde agarose gels, which were subsequently stained with ethidium bromide to determine that similar amounts of 28S ribosomal RNA were present in each sample (44). Northern blots were prepared and hybridized with nick-translated cDNA probes (~1 × $10^7$ cpm) specific for human platelet thrombospondin (starting 115 bases upstream from the start codon to 800 bases beyond the stop codon), generously provided by Dr. William A. Frazier (Washington University, St. Louis, MO), and then with a cDNA probe for chick β-actin (9) under stringent conditions (44). The blots were exposed to x-ray film for analysis of hybridization of the probes to mRNA present in the samples for 16 h at −70°C.

## Protein Labeling

Pulse–chase analysis of protein biosynthesis was performed on cells plated at an initial density of 40,000 cells/cm² in 35-mm dishes. After 24 h of

recovery, the medium was removed and the cells incubated for 48 h in the same medium but also containing 10 mM β-glycerol phosphate, ITS (insulin, transferrin, and selenium, all at 5 μg/ml) (Collaborative Research, Lexington, MA), and 100 μg/ml ascorbic acid. After a 1-h preincubation in DME without glutamine and leucine, but with 10% dialyzed FBS and other additives noted above, the cells were pulse-labeled with 10 μCi/ml each of L-[$^{14}$C(U)]leucine (337.1 mCi/mmol) and L-[$^{14}$C-(U)]proline (262 mCi/mmol New England Nuclear, Boston, MA) for 10 min. The cultures were then washed three times with complete medium, and incubated for various time points up to 12 h. At each time point, the medium and cell layer were separated and the cell layer was washed three times with PBS containing 0.01 M EDTA, and 0.01 M PMSF, and the first wash was combined with the medium fraction. The samples were stored frozen until analyzed.

## Modulation of Protein Biosynthesis by TGF-β

Cells were plated at a density of 25,000 cells/cm² and allowed to recover for 24 h in complete medium. Preliminary experiments indicated that the secretory capacity of the human bone cells is greatly reduced by incubation in serum-free medium, even for short periods of time, and consequently treatment with TGF-β was performed in the presence of 2% heat-inactivated FBS, a level of serum found to support protein secretion. The cells were washed twice in complete medium containing 2% FBS and incubated for 24 h before the addition of fresh medium containing increasing doses of TGF-β (kindly provided by Dr. Anita B. Roberts, National Cancer Institute, National Institutes of Health, Bethesda, MD) ranging from 0.01 to 10 ng/ml. After 24 h, the cultures were incubated for an additional 6 h with 5 μCi/ml each of L-[$^{14}$C(U)]leucine and L-[$^{14}$C(U)]proline as described above. At the end of the incubation, the medium and cell layer fractions were separated, and stored frozen until analyzed further.

## Immunoprecipitation of Radiolabeled Proteins

After thawing, medium and cell layer fractions were processed for immunoprecipitation in the presence of detergents using protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) as described elsewhere (25). The medium was diluted 3:1 4× immunoprecipitation buffer (1× = 0.05 M Tris-HCl, pH 7.4, 0.15 M sodium chloride, 1% Triton X-100, 1× sodium deoxycholate, 0.1% SDS, 0.1% aprotinin), and the cell layer was scraped into and extracted with 2× immunoprecipitation buffer, and subsequently diluted with an equal volume of water. Both fractions were centrifuged to remove debris. Aliquots of both medium and cell layer fractions were used for 15% TCA precipitation to determine incorporation of isotopic precursors and for electrophoretic separation of the total population of radiolabeled polypeptides. For immunoprecipitation using monoclonal mouse anti–human thrombospondin, the remainder of the sample was used for the pulse–chase analysis, and equal amounts of radioactivity were used from samples treated with and without TGF-β. After reduction of disulfide bonds with DTT, immunoprecipitates were subjected to electrophoresis on 4–20% polyacrylamide gradient gels (32) with minor modifications (18), and radioactive polypeptides visualized by fluorography (7). In some experiments, radiolabeled polypeptides were quantitated by excising them from gels and solubilizing the radioactivity and scintillation spectroscopy.

## Cell Attachment Assay

The ability of thrombospondin to mediate osteoblast cell attachment in vitro was assessed essentially as described by Roberts et al. (54). Proteins, including serum-free conditioned medium of adult human bone cell cultures, human osteopontin (1 μg/ml), purified as described previously (19), thrombospondin, kindly provided by Dr. David D. Roberts (at 10 and 50 μg/ml), and BSA (500 μg/ml) were dissolved in PBS containing 1 mM CaCl₂, and 10 μl of these solutions were applied to bacteriological dishes (resulting in a protein-coated area of 3 mm²). Each 60-mm bacteriological plate contained three "dots" of each protein tested. After a 16-h incubation at 4°C, the solutions were removed by suction and the plates were washed with 50 mM Tris-HCl, pH 7.8, 110 mM NaCl, 5 mM CaCl₂, 0.1 mM PMSF, 1% BSA for 30 min at room temperature, and then three times with serum-free DME/Ham's F12 (1:1) containing 0.5% ITS+ (insulin, transferrin, and selenium [2.5 μg/ml], BSA [500 μg/ml], and linoleic acid [0.5 μg/ml]) (Collaborative Research) with glutamine, penicillin/streptomycin, and ascorbic acid (DME/HF12). Adult human bone cells were preincubated for 3 h with DME/HF12 containing 10 μg/ml cycloheximide to inhibit protein synthesis, released by Trypsin-EDTA, washed twice, and seeded into the prepared 60-mm plates containing the 3-mm² protein-coated areas at a density of 40,000
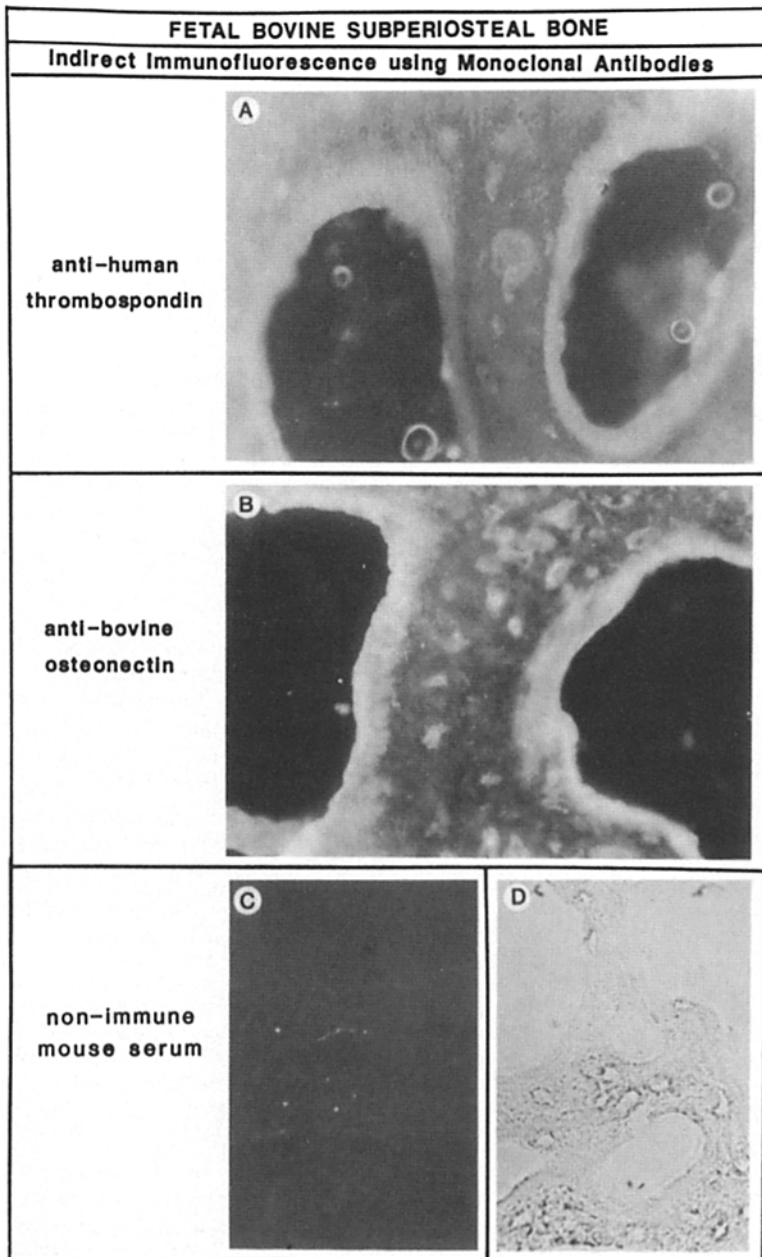
Figure 1. Detection of thrombospondin by indirect immunofluorescence. Undemineralized, frozen sections (10 $\mu$m) of fetal subperiosteal bone (D) were first reacted with either monoclonal anti–human thrombospondin (A) or anti–bovine osteonectin (B) (1:20 dilution), and subsequently with fluorescein-labeled rabbit anti–mouse IgG (1:16). The sections were viewed with a Leitz ultraviolet microscope, and photomicrographs taken using Kodak 1600 ASA film. Exposure times were 43 (thrombospondin) and 30 s (osteonectin). Sections exposed to normal mouse serum (C) were photographed for equivalent and longer exposure times and showed no reactivity.

cells/cm$^2$ in DME/HF12 containing cycloheximide. After incubation for various time intervals, the plates were washed three times with PBS to remove unattached cells, fixed with 100% methanol at $-20°$C for 30 min, and stained with Giemsa.

## Results

### Identification of Thrombospondin in Bone

Initially, the presence of thrombospondin in developing bone (Fig. 1 D) was investigated by indirect immunofluorescence (Fig. 1). Immunoreactivity with the monoclonal antithrombospondin (Fig. 1 A) was localized to the osteoid layer on the surface of mineralized matrix in fetal bovine subperiosteal bone, a pattern similar to that found using a monoclonal antibody against bovine osteonectin (Fig. 1 B). Identical results were also found for fetal human bone (data not shown). The

cells embedded in the mineralized matrix (osteocytes) were also positive, but their reactivity was not as intense as that found with antiosteonectin. The mineralized matrix was only weakly reactive with both antibodies possibly due to the presence of hydroxyapatite which blocks access if the antibody to the proteins in the mineralized bone matrix. No reaction was noted with normal mouse serum (Fig. 1 C).

Subsequently, neonatal and young bone from a variety of animal species were sequentially extracted first by dissociative, and then with dissociative and demineralizing conditions to determine if thrombospondin is a mineralized matrix protein. Thrombospondin, detected by Western blot analysis using monoclonal anti–human thrombospondin (Fig. 2), was readily apparent in human, monkey, ovine, and avian bone matrix extracts. Relatively less thrombospondin was detected in samples from bovine, porcine, canine, rat, mouse,
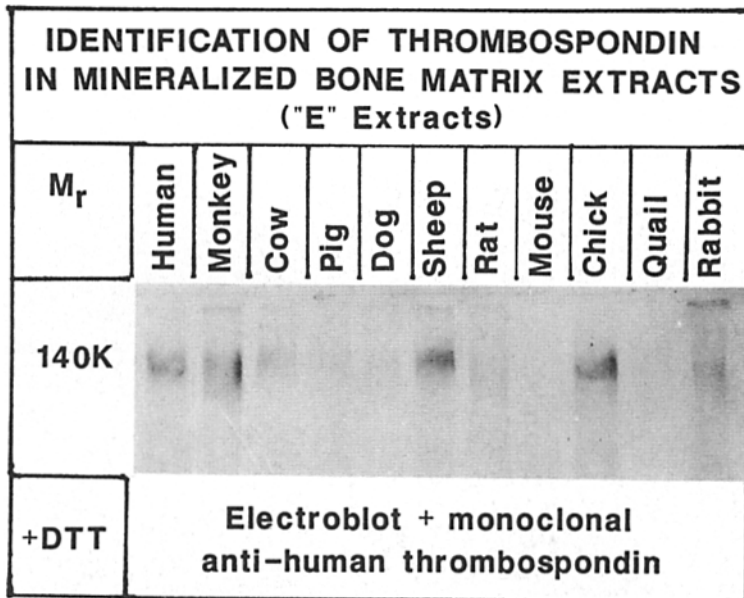
Figure 2. Identification of thrombospondin by Western blot. Mineralized matrix proteins isolated from a number of animal species as described in Materials and Methods were subjected to electrophoresis on 12% polyacrylamide gels, and transferred by electroblotting to nitrocellulose. The blot was initially reacted with monoclonal anti–human thrombospondin (1:2,000), then with peroxidase-conjugated rabbit anti–mouse IgG, and finally with peroxidase substrate.

and rabbit bone samples. However, it should be noted that it is difficult to determine absolute amounts by this analysis due to species differences. Taken together with the histochemical data, it is apparent that thrombospondin is a component of both osteoid and mineralized bone matrix.

### Identification of Thrombospondin as an Osteoblastic Cell Product

Thrombospondin is not generally found free in the circulatory system, and consequently would not adsorb to bone hydroxyapatite from serum as is the case with serum $\alpha2$-HS glycoprotein, a protein synthesized in the liver and concentrated in bone. However, it is possible that thrombospondin might be synthesized by cell types other than osteoblasts that are adjacent to bone and then entombed within bone during the mineralization process. To determine if osteoblasts contribute, at least in part, to the thrombospondin content of bone, human bone cell cultures were established and used to identify thrombospondin mRNA, and to study the biosynthesis of this molecule. Northern blot analysis using a cDNA probe specific for thrombospondin revealed a 6.1-kb mRNA in human bone cells, as well as in skin fibroblasts, and in gingival and periodontal ligament cells (Fig. 3). Interestingly, thrombospondin mRNA was not found in human decidual tissue of the uterus, although relatively equal amounts of actin mRNA and 28S ribosomal RNA were present in this sample.

In light of recent evidence that mRNA may be present but not translated by cells under certain conditions (5), the biosynthesis of thrombospondin by osteoblasts was demonstrated by pulse–chase analysis (Fig. 4). Thrombospondin monomer, detected by immunoprecipitation, was found in the pulse-labeled cell layer fraction with an apparent molecular weight of ~180,000 and was not detectably different from the secreted molecule. During the chase intervals, thrombospondin levels in the cell layer were maximal between 1 and 2 h. However, significant levels remained cell layer–associated after extended chase intervals, and ~10% of the maximal level was present after a 12-h chase, as deter-

mined by quantitation of radioactivity from solubilized gel slices. Secretion into the medium was detected after a 40-min chase, and maximum levels were achieved at 1 h and gradually fell to ~50% of the maximum level after a 12-h chase. This pattern of synthesis and secretion is somewhat slower than that of type I procollagen and osteonectin, but similar to that of fibronectin, as determined by sequential immunoprecipitation (24) of these proteins from these same samples (P. Gehron Robey, unpublished results).

### Effect of TGF-β on Thrombospondin Biosynthesis

Since TGF-$\beta$ is a regulator of matrix protein production in many cell culture systems (48, 51, 70), its effects on thrombospondin production by fetal and adult bone cells were investigated. In the presence of 2% FBS, exogenous TGF-$\beta$ does not stimulate mitogenesis by 24 h. With increasing doses of TGF-$\beta$ ranging from 0.01 to 10.0 ng/ml, total protein synthesis was slightly inhibited in both fetal and adult human bone cells (at 10 ng/ml, 78.5 $\pm$ 2.1% and 63 $\pm$ 1.4% of fetal and adult cells, respectively; $n$ = two experiments), as determined by TCA precipitation of aliquots of medium and cell layer fractions. Total thrombospondin biosynthesis, determined by quantitation of radioactive immunoprecipitated thrombospondin was marginally inhibited (10 ng/ml, 94 $\pm$ 4.2% and 88.2 $\pm$ 2.8% of fetal and adult control levels). Interestingly, the amount of thrombospondin associated in the cell layer/matrix fraction appeared to increase in TGF-$\beta$–treated samples (Fig. 5). Others have noted a marked stimulatory effect of TGF-$\beta$ on matrix protein metabolism in serum-free conditions in cells derived from other species (48, 51, 70). The biological significance of the marginal effects observed in human bone cells in the presence of 2% FBS is not yet clear.

### Effect of Thrombospondin on Osteoblast Cell Attachment

The effect of thrombospondin in mediating adult human bone cell attachment was investigated and compared to other pro-
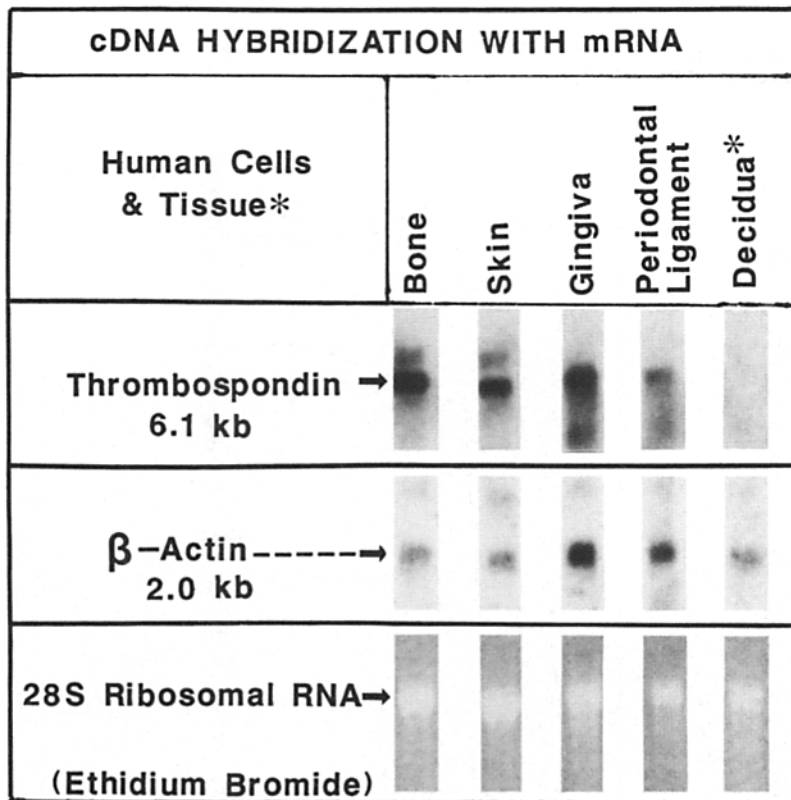
## cDNA HYBRIDIZATION WITH mRNA

| Human Cells & Tissue* | Bone | Skin | Gingiva | Periodontal Ligament | Decidua* |
|---|---|---|---|---|---|
| Thrombospondin → 6.1 kb | | | | | |
| β–Actin ------→ 2.0 kb | | | | | |
| 28S Ribosomal RNA→ (Ethidium Bromide) | | | | | |

*Figure 3.* Hybridization of mRNA with thrombospondin cDNA. mRNA (∼10 µg) was isolated and subjected to electrophoresis on 1.2% formaldehyde agarose gels. Ribosomal RNA was visualized by ethidium bromide to determine that roughly equivalent amounts of material were present in each lane. The RNA was blotted onto nitrocellulose and reacted first with human thrombospondin cDNA and then with chick β-actin cDNA probes under stringent conditions. The blots were exposed to x-ray film for 16 h at −70°C.
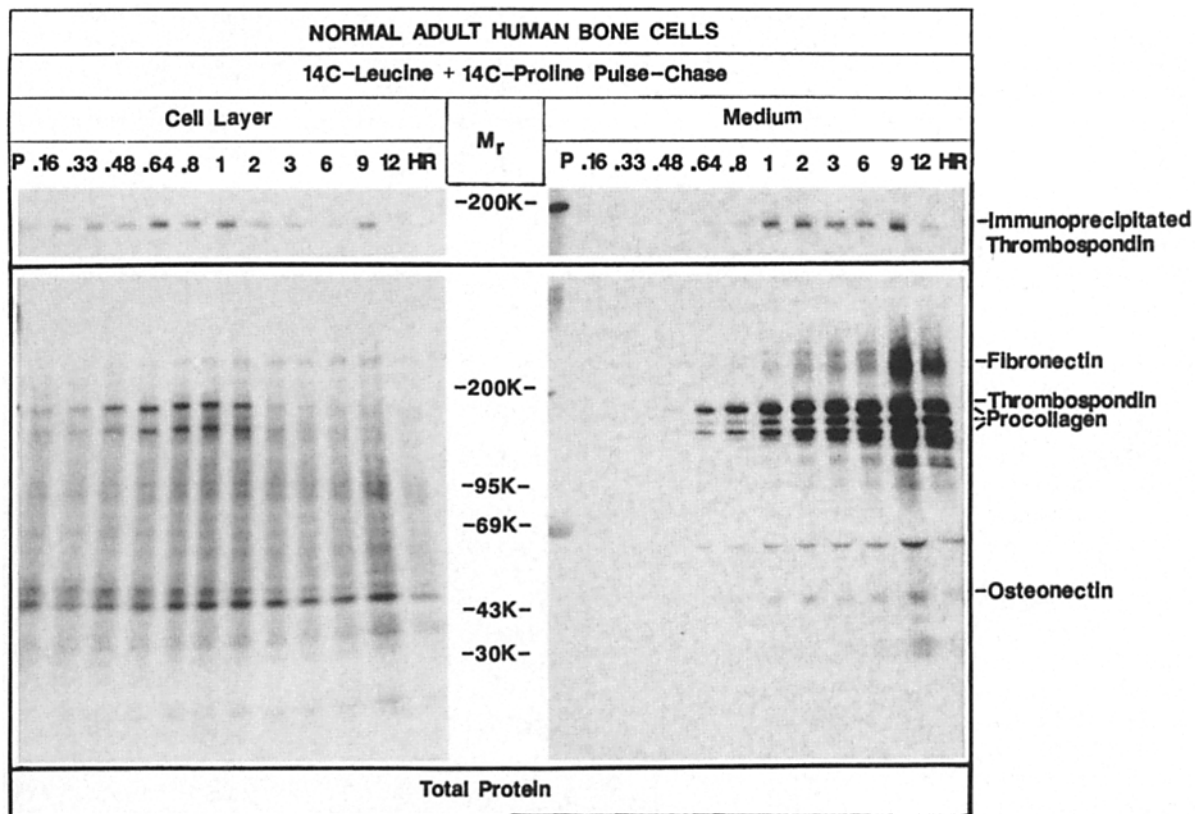
## NORMAL ADULT HUMAN BONE CELLS

### 14C–Leucine + 14C–Proline Pulse–Chase

| Cell Layer | | | | | | | | | | | | $M_r$ | Medium | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P | .16 | .33 | .48 | .64 | .8 | 1 | 2 | 3 | 6 | 9 | 12 HR | | P | .16 | .33 | .48 | .64 | .8 | 1 | 2 | 3 | 6 | 9 | 12 HR |

–200K–  –Immunoprecipitated Thrombospondin

–200K–  –Fibronectin  –Thrombospondin  –Procollagen

–95K–
–69K–  –Osteonectin
–43K–
–30K–

**Total Protein**

*Figure 4.* Pulse–chase analysis of thrombospondin biosynthesis. Cultures of adult human bone cells (50,000 cells/cm² in 35-mm dishes) were preincubated in leucine-free DME containing 10% dialyzed FBS and supplements (see Materials and Methods) for 1 h and then pulse-labeled for 10 min with 5 µCi/ml each of [¹⁴C]leucine and [¹⁴C]proline. After washing three times, the cultures were incubated from various time intervals in complete medium. Thrombospondin immunoprecipitations, as well as equal aliquots of the medium and cell layer fractions were subjected to electrophoresis on 4–20% polyacrylamide gradient gels after reduction of disulfide bonds with DTT. Radioactive polypeptides were visualized by fluorography.
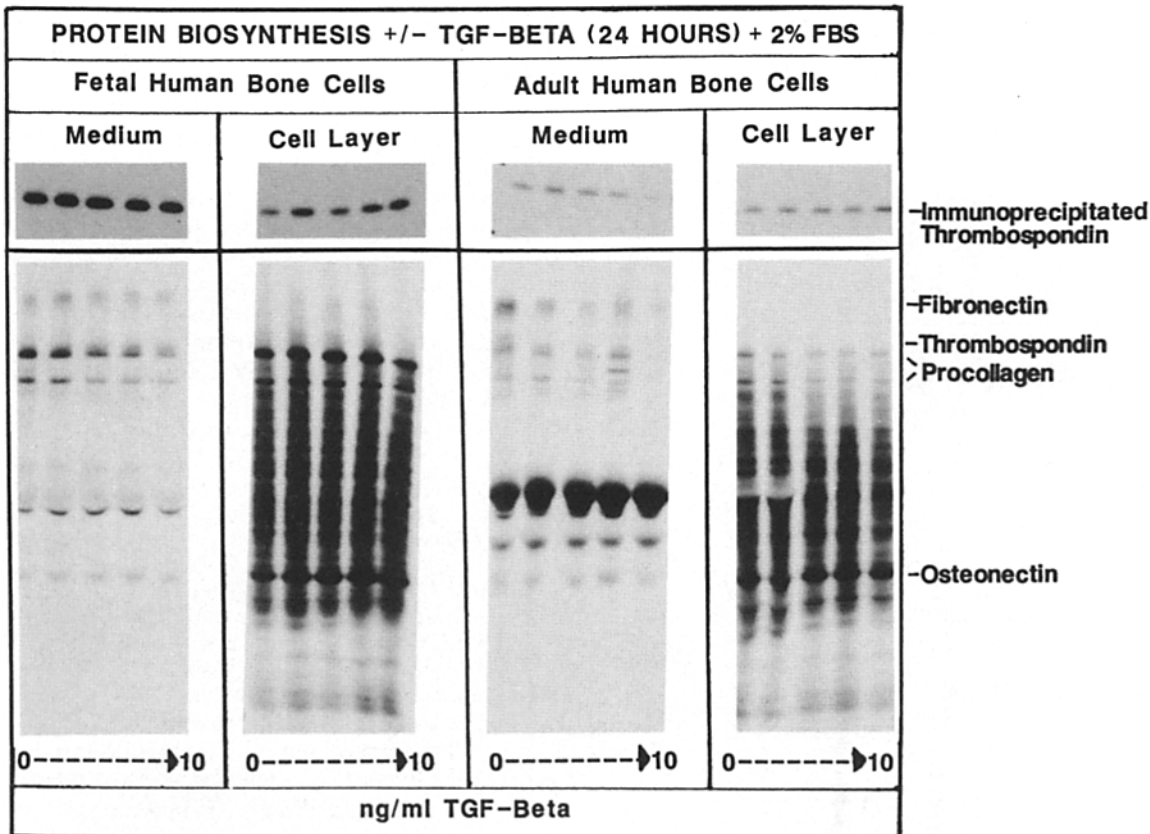
**PROTEIN BIOSYNTHESIS +/− TGF−BETA (24 HOURS) + 2% FBS**

| Fetal Human Bone Cells | | Adult Human Bone Cells | |
|---|---|---|---|
| Medium | Cell Layer | Medium | Cell Layer |

−Immunoprecipitated Thrombospondin

−Fibronectin
−Thrombospondin
〉Procollagen

−Osteonectin

0−−−−−−−▶10   0−−−−−−−▶10   0−−−−−−−▶10   0−−−−−−▶10

**ng/ml TGF−Beta**

*Figure 5.* Effect of TGF-$\beta$ on thrombospondin biosynthesis. Fetal and adult human bone cells (25,000 cells/cm$^2$ in six-well plates) were incubated in the complete medium with 2% FBS for 24 h before treatment with TGF-$\beta$ (0.01 ng, 10 ng/ml) for an additional 24 h. The cultures were then radiolabeled with 5 $\mu$Ci/ml each of [$^{14}$C]leucine and [$^{14}$C]proline in leucine-free DME with 2% dialyzed FBS and supplements (see Materials and Methods) for 6 h. Incorporation of isotope into the medium and cell layer fractions was determined by 15% TCA precipitation, and equal levels of radioactivity were used for thrombospondin immunoprecipitation and for analysis of the total population of radiolabeled polypeptides. Samples were subjected to electrophoresis on 4–20% polyacrylamide gradient gels and visualized by fluorography.

teins known to exhibit this property, including osteopontin (also known as bone sialoprotein I and 44-kD phosphoprotein). Osteopontin (49, 50) has been found to be a cell attachment protein for both a rat osteosarcoma cell line, ROS 17.0/2.8 (49), and for human periodontal ligament cells (60). Bone cells were found to attach to areas coated with serum-free bone cell–conditioned medium, osteopontin, and thrombospondin (Fig. 6). However, differences in the pattern of attachment were noted. Although similar numbers of cells attached to the various substrates (as determined by counting of cells in several fields), bone cell–conditioned medium mediated cell attachment, spreading, and migration (as noted by the development of pseudopodial processes). Cells attached to osteopontin (93.3 ± 3.2% of the number of cells attached to conditioned medium-coated areas; $n$ = three experiments), but remained ellipsoidal and relatively stationary. Cells attached in the thrombospondin-coated areas (85.5 ± 5.1%, $n$ = three experiments), but did not spread, even upon prolonged (24 h) incubation.

## Discussion

Based on the results presented here, it appears that thrombospondin is a previously unidentified component of osteoid and mineralized bone matrix. Osteoblasts would appear to contribute to bone matrix thrombospondin, since these cells contain thrombospondin mRNA and synthesize this molecule in vitro. However, other cell types associated with bone (endothelial cells, marrow stromal cells, etc.) may also contribute to this pool.

Since its initial discovery (2), a multitude of studies suggest that thrombospondin may mediate the secondary (secretion-dependent) phase of platelet aggregation (3, 14, 22, 37, 38). Due to its many interactions with other matrix molecules, another potential function(s) may lie in the organization of the extracellular matrix of connective tissue cells. It was also suggested that in certain cell types, thrombospondin can even function as an autocrine growth factor (41). The role of this molecule in bone metabolism is not known, but in light of previous studies in other cell and tissue systems, it is interesting to speculate on its potential functions in bone.

Since the major (carboxy-terminal) globular domain of thrombospondin contains multiple Ca$^{++}$ binding sites, it is possible the molecule may exert its function in bone via this property. In fact, the interaction of calcium with thrombospondin is not trivial. It has been found, for example, that the conformation of the major globular domain, in which the RGD sequence is buried, is affected by the presence or ab-
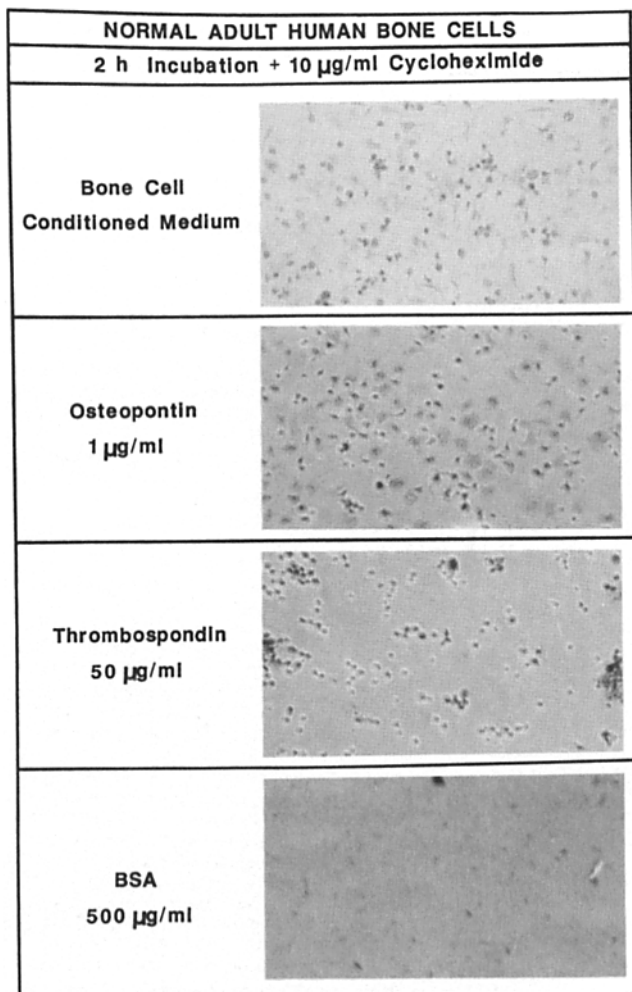
| NORMAL ADULT HUMAN BONE CELLS |
|---|
| 2 h  Incubation + 10 µg/ml  Cycloheximide |



*Figure 6.* Bone cell attachment. 10 µl of serum-free adult human bone cell–conditioned medium, osteopontin (1 µg/ml), and thrombospondin (50 µg/ml) were dotted onto 60-mm bacteriological dishes (resulting in a 3-mm² area) and allowed to adsorb at 4°C overnight. The dishes were washed as described in the text and adult human bone cells, previously incubated in cycloheximide (10 µg/ml), were added at a density of 40,000 cells/cm² in DME/HF-12 containing 0.5% ITS+ and cycloheximide. After a 2-h incubation at 37°C, the plates were carefully washed, fixed with 100% methanol at −20°C, and stained with Giemsa.

sence of Ca⁺⁺ (16, 37). In previous reports using fragments of the thrombospondin molecule, it was found that the amino terminus is involved in cell spreading but that attachment does not occur unless the carboxy-terminal globular domain is present (54). Therefore, the ability of this molecule to mediate cell attachment may be mediated by the ionic environment in which it is found. In fact, it has been found that keratinocytes grown in low calcium medium synthesize thrombospondin, and attach and spread on thrombospondin-coated substrates. However, if the calcium concentration is increased, thrombospondin synthesis and cell attachment are reduced (65). It is not yet known how Ca⁺⁺ levels regulate thrombospondin biosynthesis or cell attachment and spreading in osteoblasts.

A fascinating aspect of thrombospondin is seen in recent studies of aortic smooth muscle cells, where stimulation of proliferation by PDGF selectively induces the production of certain proteins, including thrombospondin (41, 42). Furthermore, thrombospondin synergizes with EGF to increase proliferation (41), and if thrombospondin is inactivated (by blocking monoclonal antibodies) (43) or removed from the pericellular environment (by addition of heparin which binds to thrombospondin), cell proliferation is decreased (41). In this study, it appeared that TGF-$\beta$ caused an increase in cell layer/matrix–associated thrombospondin in spite of a reduced total protein synthesis and a slight reduction in overall thrombospondin synthesis. By analogy to the aortic smooth muscle system, it is possible that the mitogenic effects of TGF-$\beta$ are mediated by thrombospondin.

Of particuar interest is the possible interaction of thrombospondin and osteonectin. Osteonectin (SPARC, BM-40, culture shock protein) has been identified in the $\alpha$ granules of platelets (61) and is released along with thrombospondin during platelet aggregation. A recent study has demonstrated that thrombospondin and osteonectin bind to one another (10). In many species, including man, osteonectin is the most abundant noncollagenous protein in bone. Although osteonectin is 100–1,000 times more concentrated in bone compared to other connective tissues, it appears to be identical to proteins that have been identified in other tissues. It is synthesized by certain tissues during various stages of murine development (26), by certain basement membrane forming cells (17, 66), and in response to varying culture conditions (56). In general, the expression and accumulation of osteonectin in nonbone sources in vivo, appears to be transient (presumably induced and repressed by local factors), and related to stages of proliferation and/or maturation, whereas in bone, expression appears to be constitutive and the protein remains associated with extracellular matrix. The significance of the colocalization of thrombospondin and osteonectin in such different tissue systems (platelets and bones) is not yet known, but it is possible that these two molecules (along with others) create an environment that is conducive for remodeling (e.g., wound repair), as particularly exemplified by bone, which is in a constant state of remodeling (bone turnover).

Clearly, much more investigation is needed to define the role of thrombospondin in bone, but the hints that nature has provided (colocalization of thrombospondin and osteonectin in platelets and bone) provide a direction for future comparative studies to identify common mechanisms in these two tissues that may elucidate the functions of these interesting molecules.

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