

A Potential Role for Tetranectin in Mineralization during Osteogenesis

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Abstract. Tetranectin is a protein shared by the blood and the extracellular matrix. Tetranectin is composed of four identical, noncovalently bound polypeptides each with a molecular mass of ~ 21 kD. There is some evidence that tetranectin may be involved in fibrinolysis and proteolysis during tissue remodeling, but its precise biological function is not known. Tetranectin is enriched in the cartilage of the shark, but the gene expression pattern in the mammalian skeletal system has not been determined. In the present study we have examined the expression pattern and putative function of tetranectin during osteogenesis. In the newborn mouse, strong tetranectin immunoreactivity was found in the newly formed woven bone around the cartilage anlage in the future bone marrow and along the periosteum forming the cortex. No tetranectin immunoreactivity was found in the proliferating and hypertrophic cartilage or in the surrounding skeletal muscle. Using an *in vitro* mineralizing system, we examined osteoblastic cells at different times during their growth and differentiation. Tetranectin mRNA appeared in the cultured osteoblastic cells in parallel with mineralization, in a pattern similar to

that of bone sialoprotein, which is regarded as one of the late bone differentiation markers. To explore the putative biological role of tetranectin in osteogenesis we established stably transfected cell lines (PC12-tet) overexpressing recombinant tetranectin as evidenced by Northern and Western blot analysis and immunoprecipitation. Both control PC12 cells and PC12-tet cells injected into nude mice produced tumors containing bone material, as evidenced by von Kossa staining for calcium and immunostaining with bone sialoprotein and alkaline phosphatase antiserum. Nude mice tumors established from PC12-tet cells contained approximately fivefold more bone material than those produced by the untransfected PC12 cell line or by the PC12 cells transfected with the expression vector with no insert (Mann Whitney rank sum test, $p < 0.01$), supporting the notion that tetranectin may play an important direct and/or indirect role during osteogenesis. In conclusion, we have established a potential role for tetranectin as a bone matrix protein expressed in time and space coincident with mineralization *in vivo* and *in vitro*.

TETRANECTIN is a protein originally purified from human plasma that was found to be composed of four identical, noncovalently bound polypeptides (14, 16). The cDNA of human tetranectin has been cloned (40) and its genomic structure revealed (3). The deduced amino acid sequence consists of 202 amino acids including a classical hydrophobic 21-amino acid signal sequence. The molecular mass of the tetranectin monomer is calculated to be 20,169 kilodaltons. In accordance with the size of the cDNA, Northern blot analysis reveals an ~ 1 kb¹ mRNA signal. Tetranectin shows sequence homology to asialoglycoprotein receptors, cartilage proteoglycan core protein and a number of surfactant factors (3, 16, 40). Tetranectin binds to the

kringle four domains of plasminogen (14) and apolipoprotein(a) (25) and also binds to fibrin (26), calcium (14), heparin (14), and other sulfated polysaccharides (13).

Immunohistochemically, we and others have found that tetranectin is located in the extracellular matrix of human carcinomas, while little or no immunoreactivity could be demonstrated in the extracellular matrix of the corresponding normal tissues (11, 40). Tetranectin mRNA is upregulated in the stroma of colon cancer (40). The molecular mechanism of this upregulated expression pattern in tumoral stroma, which is reminiscent of that of tenascin (8) remains unknown. The function of tetranectin in the stroma during tumorigenesis is not presently understood.

Recently, the primary structure of tetranectin from reef shark (*Carcharhinus springeri*) cartilage (30) was determined. In shark, tetranectin was found to be one of the main non-collagenous, non-proteoglycan components of the neural arch cartilage. This led us to explore the expression and

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putative role of tetranectin in the mammalian skeletal system. In the present study, we have found that tetranectin gene expression during bone development appeared in time and space coincident with mineralization *in vivo* and *in vitro*. We established stably transfected PC12 cell lines (PC12-tet) and tumors generated from cells expressing tetranectin promoted bone formation to a greater extent than control cell lines (PC12 untransfected and PC12 transfected with the expression vector with no insert), strongly indicating a role for tetranectin during osteogenesis.

Materials and Methods

Cells and Culture Conditions

A novel *in vitro* culture system from primary bovine osteoblast-like cells has recently been established in which a thick mineralized matrix is eventually formed (42). Since the expression patterns of some extracellular matrix proteins during mineralization were determined in this system (23), tetranectin expression was studied in this system. Briefly, the osteoblast-like cells were isolated from 5-month-old fetal bovine long bones and cultured as described (23, 42). For the mineralization assays, cells were seeded at a density of 300,000 cells per 35-mm culture dish and grown for 3–5 d. Then the growth medium was exchanged with mineralization medium (designated day 0) which contained 10 mM beta-glycerophosphate, 15 mM Hepes, 50 µg/ml ascorbic acid, and 0.12 ml ITS (insulin, transferrin, and selenium; Sigma Chem. Co., St. Louis, MO). The cultures were maintained in mineralization medium up to 20 d. The WI-38 human embryonal lung fibroblasts (ATCC CCL 75) and COS-7 (ATCC CRL 1651) were obtained from the American Type Culture Collection, Rockville, MD. The CHO cells were kindly provided by Drs. J. Verheijen and C. Kluft, Leiden, The Netherlands, and the BAE bovine endothelial cells by Dr. A. M. Mercurio (Boston, MA). These cell lines were routinely maintained in DMEM with Glutamax I and 4,500 mg/ml glucose, penicillin and streptomycin, and 10% FCS (growth medium). The PC12 cells, which are derived from a rat pheochromocytoma (19), were kindly provided by Dr. A. E. Chung, (Pittsburgh, PA) and maintained as described (33).

Antibodies

Polyclonal antibodies against tetranectin purified from human serum (code A371, lot 096, 0.075 mg Ig/ml) and normal rabbit serum (code 902, lot 012) were purchased from DAKO a/s. These antibodies have been characterized and used in several studies (i.e., 10–12). Von Kossa staining for calcium (34), polyclonal antibodies (LF-87) to bone sialoprotein (29) kindly provided by Dr. L. Fisher, National Institutes of Health, and antibodies to alkaline phosphatase (21) kindly provided by Dr. J. L. Millan (La Jolla Cancer Research Foundation), were applied to confirm bone formation.

Tissues and Immunostaining

Newborn mice (C57/BL) bred in our animal facilities were sacrificed and tissues collected, fixed in formalin, embedded in paraffin, cut, and processed for immunoperoxidase staining essentially as described (40). Before immunostaining, the sections were treated with 2% H₂O₂ in methanol for 10 min to abolish endogenous peroxidase, and for the tetranectin immunostainings, in addition, treated with 1 mM tranexamic acid (Sigma A-6516) in 0.05 M Tris-HCl for 5 min at room temperature. As a control for the tetranectin immunostaining of the tissue sections, normal rabbit serum was used or competition assays were performed as follows. Tetranectin antibodies diluted 1:50 were incubated together with recombinant tetranectin (culture medium supernatant of PC12-tet cells) in a 1:1 ratio overnight at 4°C, and the immunostaining was performed as described above. As a result only faint or no immunoreactivity was observed. When we used the untransfected PC12 cell supernatant, only a slight reduction in the intensity of the immunoreactivity was observed; this was most likely due to the presence of immunoreactive tetranectin in FBS.

Construction of a Tetranectin Expression Vector

Primers were selected for PCR based on the sequence information obtained by cDNA cloning of human tetranectin (40) and synthesized in our laboratory on an ABI model 3808 DNA synthesizer using phosphoramidite chem-

istry. A PCR product containing the full-length coding sequence starting at the ATG (nt 94) and ending at the stop codon TAG (nt 702) was obtained, using 5' end sense primer with a clamp and a Hind III site (5'-ATCT-AAGCTTATGGAGCTCTGGGGGGCCTA) and 3' end reverse complementary primer with a clamp and a Xba I site (3'-TCTCTCTAGACTACCGAT-CCCGAACT). The original tetranectin plasmid (designated TET-1) was used as template and conditions were as described (40). The PCR product was subsequently treated with restriction enzymes (Boehringer Mannheim Corp., Indianapolis, IN), gel-purified and subcloned into the Hind III-Xba I site of the pRc/CMV expression vector (In Vitrogen, San Diego, CA), generating the recombinant plasmid pRc/CMV-tet. This expression vector contains the immediate early gene promoter of human cytomegalovirus (CMV)¹ for high level transcription, the polyadenylation signal and transcription termination sequences from the bovine growth hormone gene, and the neomycin resistance gene for selection of G-418 resistant stable cell lines. Highly purified plasmid was obtained as described (36). Sanger DNA sequencing confirmed the correct reading frame of the PCR product in the recombinant expression vector (pRc/CMV-tet).

Transfection of Tetranectin

For transient transfections subconfluent (~80% confluent) COS-7, CHO, WI-38, BAE, and PC12 cells were rinsed twice in serum-free Opti-MEM (GIBCO BRL, Gaithersburg, MD) and were transfected with a 2 µg of CsCl-banded supercoiled DNA (pRc/CMV-tet or pRc/CMV alone as a control) in the presence of Lipofectin Reagent according to the manufacturer's protocol (GIBCO BRL). After 6 h of incubation at 37°C, the DNA solution was removed and replaced with the growth medium for these cells, as described above. Cells were fixed for immunofluorescence 2 d after transfection. For production of stably transfected cell lines, PC12 cells were used. The cells were seeded in 60-mm tissue culture dishes and transfected with 5 µg DNA in the same fashion as described for transient transfections. Two days later, the transfectants were selected by adding G-418 sulphate (Geneticin, GIBCO BRL) at a final active concentration of 374 µg/ml. Resistant cells were further cloned by the limiting dilution method. Fifteen cell lines (PC12-tet) transfected with the pRc/CMV-tet plasmid and five cell lines (PC12-control) transfected with the pRc/CMV control plasmid were selected.

RNA and Northern Blot Hybridization

Extraction, isolation, and Northern blot analysis of the bovine osteoblast-like cells was performed as described previously (23). Briefly, the cells were rinsed in PBS, homogenized in 4 M guanidine-thiocyanate, 25 mM acetate, pH 5.4, and 0.12 M β-mercaptoethanol and subjected to density gradient centrifugation. For Northern blot analysis, aliquots of 5 µg of total RNA for each time point were separated on 1.2% denaturing agarose gel and transferred to Optibind membrane (Schleicher and Schuell, Keene, NH).

Total RNA was isolated from the various PC12 cell lines (18) and from tissues of newborn mice and nude mice tumors (9) and used for Northern blot analysis as described (36, 40).

SDS-PAGE and Western Blot

Culture medium supernatant (200 µl/lane) was concentrated using filters (Amicon, Beverly, MA). Human serum was obtained from the investigators and 1 µl was applied per lane. Samples were separated on SDS-PAGE according to Laemmli (27) using 14% resolving gels and 4% stacking gels and transferred to nitrocellulose paper (BA85, Schleicher and Schuell). Western immunoblotting was performed as described (40).

Immunoprecipitation

Subconfluent cells in 25-cm² flasks were rinsed once with PBS and labeled in 3 ml of methionine-free DMEM (GIBCO BRL) containing 0.292 mg/l L-glutamine (1/10 of normal concentration), 1% dialysed FCS and 50 µCi/ml L-[³⁵S]methionine (SJ 1305, Amersham Corp., Arlington Heights, IL) for 6 h. Tween-20, PMSF and aprotinin were added to give final concentrations of 0.05%, 1 mM and 10 µg/ml, respectively, any debris removed by centrifugation (1,000 g, 5 min, 4°C), and the medium used directly for immunoprecipitation (41). Total labeled proteins were precipitated from the cell layer with 10% trichloroacetic acid (TCA) and dissolved in sample buffer. Labeled proteins were separated on 14% SDS-polyacrylamide gels,

1. *Abbreviations used in this paper:* CMV, human cytomegalovirus; H&E, hematoxylin and eosin.

fixed, and stained with Coomassie blue, destained, rinsed in H₂O, impregnated with En³Hancer (New England Nuclear, Boston, MA), dried, and exposed to an X-Omat AR film (Kodak) at -80°C overnight with Cronex intensifying screens (New England Nuclear).

Heterotransplantation to Nude Mice

For a generation of solid tumor tissue, 10⁷ PC12, PC12-control, or PC12-tet cells were injected subcutaneously in the back of NMRI-nu 6-wk-old female nude mice (Bom mice, Bombholtgaard Breeding and Research Centre Ltd., RY, Denmark). Aliquots of tumor tissues were serially transplanted subcutaneously into at least five animals per cell line per passage. The subcutaneous tumor tissues were fixed in buffered formalin for 24 h and processed for embedding into paraffin. Sections (4 μm) were cut and stained with hematoxylin and eosin (H&E). The H&E stained sections from tumors of seven PC12 mice, seven PC12-control, and eight PC12-tet mice (passage 2) were analyzed using the Mocha Image Analysis Software (Jandel Scientific, San Rafael, CA). The following parameters were quantitated: the average area of the tumor (in mm²), the number of sections with osteofoci out of total sections analyzed, the average size of osteofoci (in mm²), and the ratio of the area of osteofoci per area of tumor. Statistical analysis was performed using the chi-square test or the Mann-Whitney rank sum test.

Results

Tetranectin Gene Expression during Osteogenesis In Vivo

To determine whether tetranectin synthesis was correlated with osteogenesis in mammals, we examined the expression of tetranectin during bone development using immunohistochemistry and Northern blot analysis. Sections of a limb with bone formation in a newborn mouse are demonstrated in Fig. 1, A-C. Tetranectin immunoreactivity was found at locations of the newly formed woven bone. The bone tissue laid down on the fibrous surface of the cartilage anlage (the periosteum) in the midportion of the future bone exhibited strong immunoreactivity with antibodies to tetranectin. Strong tetranectin immunoreactivity was found in the bone laid down around the calcified cartilage cores in the area which becomes the future marrow space. These structures are called the primary spongiosum or primary trabeculum and the bone material is laid down by the surrounding osteoblasts. No tetranectin immunoreactivity was found in

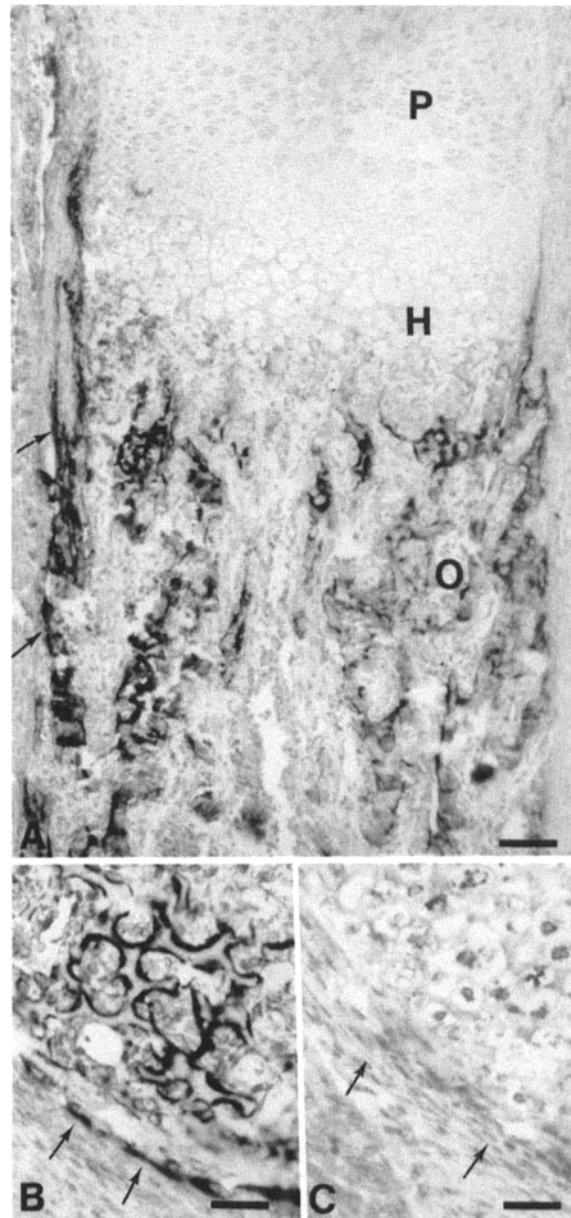


Figure 1. Tetranectin distribution during bone formation in vivo. Immunostaining (A-C) with tetranectin antibodies was performed as described in Materials and Methods. A longitudinal section of the midportion of a bone from a newborn mouse is shown. (A) Columns of proliferative chondrocytes (P), hypertrophic swollen chondrocytes (H), and their surrounding matrices do not show tetranectin immunoreactivity. In the zone of ossification (O), the woven bone laid down by osteoblasts on the surface of a core of cartilage shows strong tetranectin immunoreactivity. The woven bone laid down along cartilage cores at the periosteum, also shows intense tetranectin immunoreactivity (arrows). This is the future cortex of the bone. The surrounding skeletal muscle and connective tissue show no tetranectin immunoreactivity. (B) Larger magnification of an area with tetranectin-positive bone laid down on the cores of cartilage. Again arrows point to the bone formed at the periosteum (C). Negative control in which the primary antibodies were incubated together with recombinant tetranectin-containing PC12-tet supernatant. Arrow points the periosteum with no immunoreactivity. (D) Tetranectin mRNA expression during bone formation in vivo of a newborn mouse by Northern analysis (in duplicate, lanes 1 and 2). Liver tissue mRNA isolated and analyzed in

parallel by Northern analysis (in duplicate, lanes 3 and 4). The 1-kb message size of tetranectin is indicated. Control actin hybridization is shown below. Magnification: A, 105; B and C, 260. Bars: (A) 76 μm; (B and C) 31 μm.

the proliferating and hypertrophic chondrocytes or in the cartilage extracellular matrix. The surrounding skeletal muscle tissue showed no immunoreactivity. Controls in which the primary antibodies were replaced with normal rabbit serum showed no immunoreactivity (not shown) and the competition assays with recombinant tetranectin were also negative (Fig. 1 C). A similar pattern of tetranectin gene expression was observed in the rat and human developing bone (unpublished data). The presence of tetranectin mRNA extracted from parallel newborn mouse limbs was verified by Northern blot analysis (Fig. 1 D).

Tetranectin Expression in Cultured Bone Cells

To examine the role of tetranectin in osteogenesis, we analyzed the expression of tetranectin in an in vitro bovine mineralizing system (23, 42). In this system, the cultured cells formed multilayers and visible thick cell foci on days 2–3 after switching growth medium to mineralizing medium (day 0). Mineral deposition occurred on day 4–6 and a thin bone sheet was eventually formed between days 10–20. Fig. 2 shows that tetranectin mRNA appeared on day 6. The mRNA level increased with time and maximum expression was found on the last day of the 20-d culture period examined. The expression of tetranectin mRNA correlated with the accumulation of mineral. This expression pattern is similar to that of bone sialoprotein, which also seems to parallel mineralization and osteoblastic differentiation (23). Although their expression patterns are similar, tetranectin mRNA increased up to eightfold, while bone sialoprotein increased 140-fold. The mRNA of another bone matrix protein, decorin, increased to approximately similar extent (4.5-fold) as tetranectin, but it was detectable already on day 0.

Expression of Recombinant Tetranectin

An expression plasmid, designated pRc/CMV-tet, was constructed that contained the full-length coding sequence including the signal peptide. To test the ability of the construct to encode recombinant tetranectin, a series of cell lines (COS-7, CHO, WI-38, BAE, and PC12) were transiently transfected. In all transiently transfected cell lines intense cytoplasmic immunofluorescence reactivity was found with polyclonal tetranectin antibodies while cells transfected with the expression vector with no insert showed no immunoreactivity. No immunostaining was detected in either transfected or untransfected cells when normal rabbit serum replaced the tetranectin antiserum. Fig. 3, A and B shows an example of the immunostaining in the CHO cells. For a generation of stably transfected cell lines, we chose PC12 cells because this cell line had previously been successfully used for transfections with the extracellular matrix protein, laminin B1 chain (33). The PC12 cells were stably transfected with a recombinant plasmid pRc/CMV-tet, and fifteen neomycin resistant clones (PC12-tet) were isolated and examined for the expression of recombinant tetranectin by Northern and Western analysis. Untransfected PC12 cells and PC12-control (five selected lines) served as controls. No apparent morphological changes were observed in the transfected cells. Northern analysis of total RNA isolated from the stably transfected PC12-tet cells gave a strong hybridization signal after an overnight exposure (Fig. 3 C, lanes 1–7),

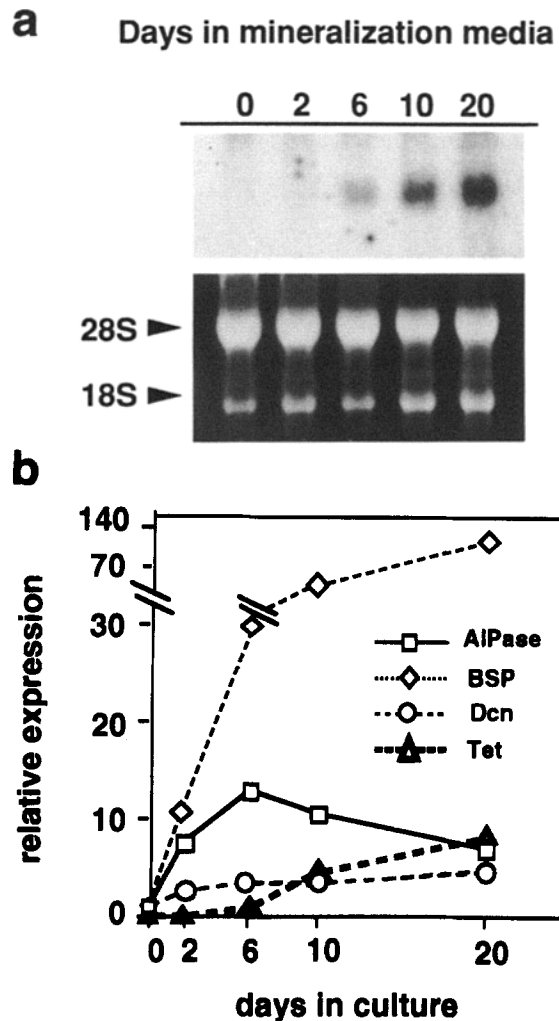


Figure 2. Tetranectin mRNA at the time of mineralization in vitro. (a) Autoradiogram of mRNA extracted from osteoblast-like cells at different stages of mineralization and probed with a ^{32}P -labeled human tetranectin cDNA. A photograph of the corresponding gel stained with ethidium bromide is shown below. The position of the 18 S and 28 S ribosomal RNAs is indicated by arrows. (b) Quantitation of the relative levels of mRNA of tetranectin (Δ), alkaline phosphatase (\square), bone sialoprotein (\diamond), and decorin (\circ). The relative amount of tetranectin mRNA was determined by densitometric scanning of the autoradiogram, and compared to the relative amounts of the other mRNAs (23) as described in Materials and Methods.

whereas no signal was found in the PC12 cells (Fig. 3 C, left panel, lanes 8–9) or in the five PC12-control cell lines (Fig. 3 C, right panel, lanes 1–5). The amount of tetranectin mRNA expression varied somewhat among the tetranectin transfected cell lines. The size of tetranectin mRNA was ~ 1 kb consistent with previous data (3, 40). In Western blot using culture medium supernatant from the transfected PC12-tet cells, tetranectin could be demonstrated as a single band whereas no immunoreactivity was observed in the non-transfected cell lines (Fig. 3 D). The molecular mass of the recombinant tetranectin monomer (21 kD) was found to be identical to human serum tetranectin as judged by the electrophoretic mobility under reducing conditions (not shown).

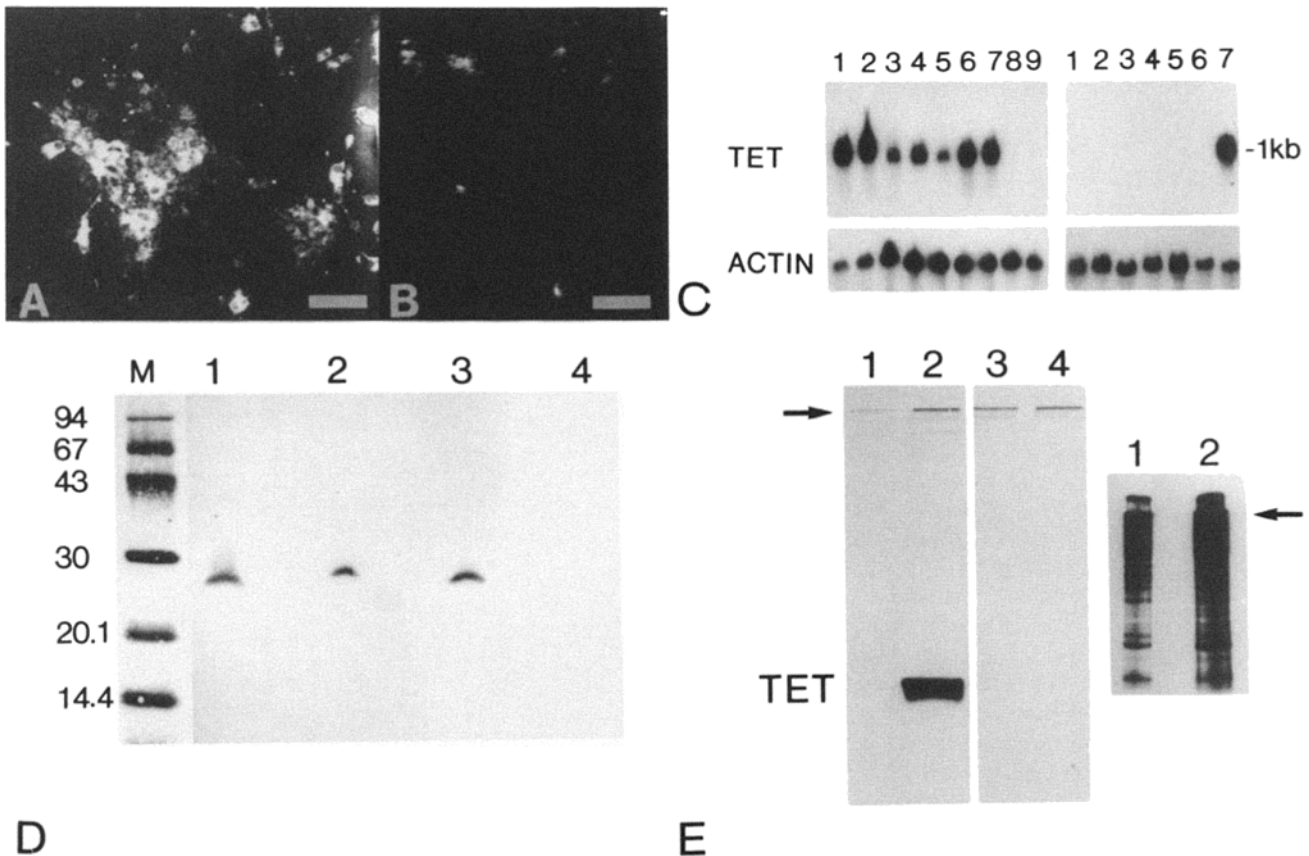


Figure 3. Establishment of tetranectin-producing PC12 cell lines (PC12-tet) (A and B). Localization of recombinant tetranectin in transiently transfected cells. The CHO cells were transiently transfected with the tetranectin cDNA expression vector pRc/CMV-tet (A) or with the control plasmid pRc/CMV (B). Cells grown in Nunc slides were briefly rinsed in 0.05 M Tris-HCl, pH 7.2, fixed in PBS-buffered formalin for 2 min at room temperature, and rinsed three times in 0.05 M Tris-HCl, pH 7.2. The cells were subsequently treated with 1% NP-40 in the Tris-HCl buffer for 1 min and with 1% Triton X-100 and 1% BSA in the Tris-HCl buffer for 2 min. Tetranectin antibodies were diluted 1:100 and normal rabbit serum 1:200 in the Tris buffer and incubated with the cells for 2 h at room temperature. After three rinses in 0.05 M Tris-HCl containing 0.15 M NaCl, the cells were incubated with swine anti-rabbit FITC secondary antibody (F205, DAKO) diluted 1:50 for 1 h at room temperature. The cells were rinsed three times in 0.05 M Tris-HCl and briefly in distilled water followed by 99% ethanol. Finally, the slides were air dried, mounted in glycerol (DAKO), and examined using a LSM-10 laser scanning microscope (Zeiss). (C) Expression of tetranectin mRNA by stably transfected PC12 cell lines. Seven independent PC12 cell lines (PC12-tet) stably transfected with the tetranectin cDNA expression vector, pRc/CMV-tet (lanes 1-7), and the non-transfected PC12 cells (lanes 8-9) were examined by Northern blotting (left panel). Another Northern blot (right panel) demonstrates 5 PC12 cell lines transfected with the pRc/CMV plasmid (lanes 1-5), the non-transfected PC12 cell line (lane 6), and one of the positive PC12-tet cell lines (lane 7). 15 μ g of total RNA was applied per lane. The filter was hybridized to the human tetranectin cDNA (upper panel) and exposed to x-ray film for 4 d. The filter was rehybridized to actin (lower panel) and exposed overnight. The 1-kb message size of tetranectin is indicated. (D) Western blot analysis of recombinant tetranectin produced by three stably transfected PC12-tet cell lines (lanes 1-3) compared to untransfected control PC12 cells (lane 4). Culture medium supernatants were analyzed by SDS-PAGE under reducing conditions, transferred to nitrocellulose, and immunostained with antiserum to tetranectin. Positions of reduced, electrophoresed, transferred, and amido black stained molecular mass standards (M in kD) are: phosphorylase b 94,000; BSA 67,000; ovalbumin 43,000; carbonic anhydrase 30,000; soybean trypsin inhibitor 20,100; and α -lactalbumin 14,400. (E) Biosynthesis of recombinant tetranectin by transfected cells. Left panel shows aliquots of [³⁵S]methionine-labeled conditioned media of PC12 (lane 1) and PC12-tet (lane 2) immunoprecipitated with tetranectin antibodies or as a control with normal rabbit serum (lanes 3 and 4). The immunoprecipitates were analyzed by SDS-PAGE under non-reducing conditions and fluorographed. PC12-tet cells which had been transfected with the tetranectin cDNA expression vector, pRc/CMV-tet synthesized an approximately M_r 21-kD monomer (lane 2, marked TET). The right panel shows TCA-precipitated material from PC12 (lane 1) and PC12-tet cells (lane 2) demonstrating that the secreted proteins from both cell lines were labeled to the approximate same extent. The arrows indicate the top of the separating gels. Bars, 50 μ m.

Biosynthesis was furthermore documented by immunoprecipitation of L-[³⁵S]methionine metabolically labeled PC12-tet cell culture supernatants with tetranectin antiserum and is shown in Fig. 3 E. A single strong 21-kD band was precipitated from the medium of PC12-tet cells (lane 2) whereas the medium of untransfected cells (PC12) showed no bands (lane

1). Control nonimmune serum did not precipitate any labeled proteins (lanes 3 and 4). No other molecular mass bands were observed even after prolonged exposure neither under nonreducing nor reducing conditions (not shown). These data show that by SDS-PAGE recombinant tetranectin was produced by the PC12-tet cells as a 21-kD monomer as

would be expected based on the size of the tetranectin cDNA and previous Northern blot analysis (3, 40). Thus, the 21-kD recombinant tetranectin monomer synthesized by PC12-tet cells appeared to be identical to plasma tetranectin.

Increased Bone Formation in Tumors Produced by Cells Transfected with Tetranectin

Nude mice were injected subcutaneously with PC12, PC12-control, and PC12-tet cell lines, and all produced tumors to the same extent. Northern blot analysis of RNA extracted from tumors showed that tetranectin mRNA was present in tumors produced by PC12-tet cells but not detectable under the same conditions in tumors produced by the PC12 cells (Fig. 4 A) or by the PC12-control (not shown). Paraffin sections from tumors were examined at the light microscopic level. H&E-stained sections of tumors from both PC12, PC12-control, and PC12-tet cells contained foci of bone material by morphological criteria (Fig. 4, B and C). In parallel sections, the bone foci stained positively for calcium by the von Kossa method (Fig. 4 D) and with antibodies to alkaline phosphatase and bone sialoprotein (not shown), verifying that the material was bone. The bone material stained positively with antibodies for tetranectin (not shown). The tumor nodules were surrounded by dense fibrous tissue, and isolated strands of skeletal muscle were often seen. Bone and cartilage were usually located at the periphery of the tumor nodules, interposed between fibrous tissue and PC12 tumor tissue. Much less bone and cartilage were located in the more central part of the tumor. This morphologic pattern was as described previously for some epithelial cell lines inducing bone formation when injected into nude mice (1, 43). By morphological examination tumors produced by PC12-tet cells appeared to contain significantly more bone material than those produced by the untransfected PC12 cells or by PC12-control cells, by several parameters that were quantitated by Mocha Image Analysis Software and LSM laserscan microscopy (Table I). First, among 121 sections examined of control mice injected with PC12 or PC12-control cells, approximately half of them had osteofoci, while all sections examined of mice injected with PC12-tet cells had osteofoci; and this difference was statistically significant (chi-square test; $p < 0.0001$). Second, the average size of osteofoci in tumors generated by PC12-tet cell was double that of the control tumors (Mann-Whitney rank sum test; $p < 0.05$). Third, the area (measured in mm²) of bone formation in tumors generated by the tetranectin-transfected cells was approximately five times larger than that of the control cells. Statistical analysis demonstrated that the difference was highly significant (Mann-Whitney rank sum test; $p < 0.01$). These findings, together with the appearance of tetranectin protein and mRNA during bone formation in vivo and in vitro, strongly indicate that tetranectin plays a role in osteogenesis.

Discussion

The data presented in this paper suggest a previously unexpected role for tetranectin in osteogenesis. Tumors produced in nude mice by PC12 cells transfected with a tetranectin cDNA expression vector contained approximately five times as much bone material compared to tumors produced by non-transfected control cells or PC12 cells transfected with the expression vector with no insert. Furthermore, during

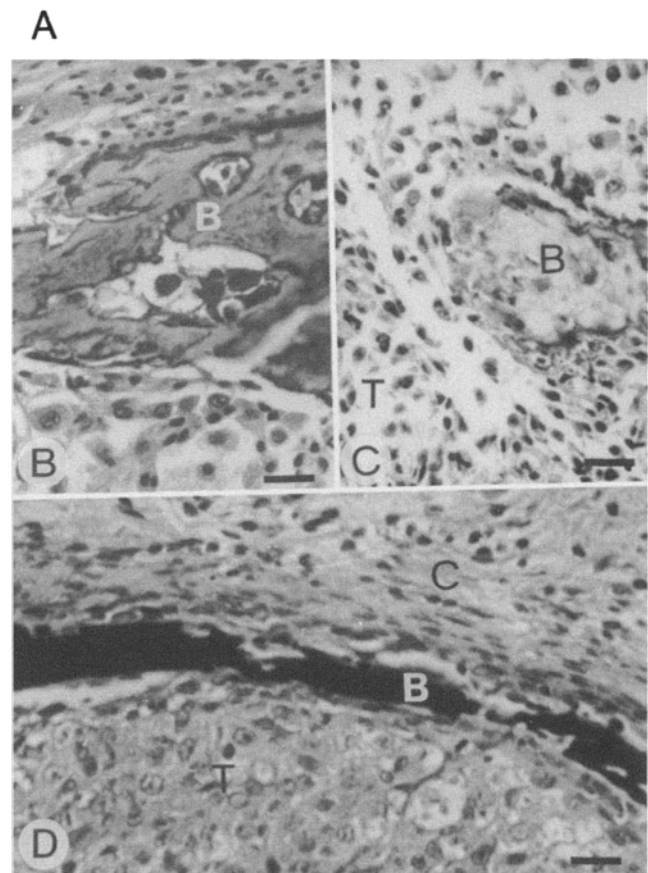
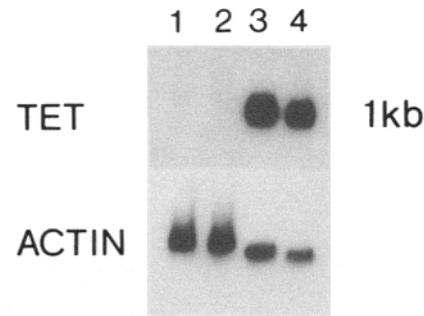


Figure 4. Nude mice tumors with bone formation. (A) Expression of tetranectin mRNA in nude mice tumors. Total RNA (15 μ g/lane) isolated from nude mice tumors of PC12 untransfected control cells (lanes 1 and 2) and PC12-tet cells transfected with tetranectin cDNA expression vector (lanes 3 and 4) were analyzed by Northern blotting. The filter was hybridized to the human tetranectin cDNA (upper panel) and exposed to x-ray films for 3 d. The filter was re-hybridized to actin (lower panel) and exposed overnight. The 1-kb message size of tetranectin is indicated. (B) Morphologic analysis of H&E stained paraffin sections of nude mice tumors generated by PC12-tet cells compared to the untransfected PC12 cells, shown in C. Bone material is the homogeneous, acellular material. In parallel sections of the PC12-tet tumors, von Kossa staining for calcium verifies the presence of bone, shown in D. Symbols on the figures indicate: T, tumor; B, bone; C, connective tissue. Magnifications: (B and C) 340; D, 160. Bars: (B and C) 24 μ m; (D), 50 μ m.

mineralization in vitro, the expression of tetranectin mRNA was induced and increased during the course of mineralization. This observation is consistent with the finding that tetranectin immunoreactivity and mRNA are strongly ex-

Table 1. Quantitation of Bone Material in Nude Mice Tumors Generated by Injection of PC12-tet Cells Compared to Two Sets of Controls, Untransfected PC12 Cells and PC12 Cells Transfected with the Expression Vector with No Insert

	Number of cases	Average mm ² of tumor specimen	Number of sections with osteofoci out of total sections analyzed	Average size of osteofoci in mm ²	Average mm ² osteofoci per mm ² tumor
PC12 controls*	14	14.24 ± 4.37	51/121	1.01 ± 0.89	0.03 ± 0.03
PC12-tet	7	14.28 ± 2.99	25/25†	2.27 ± 1.67‡	0.16 ± 0.14‡

* The controls include untransfected PC12 cells and PC12 cells transfected with the expression vector with no insert. There were no differences between the two types of control cell lines and they were therefore combined for statistical analysis.

† All sections from tumors generated by PC12-tet cells contained osteofoci whereas only approximately half of the sections from tumors generated by control PC12 cells contained osteofoci. Statistical analysis using the chi-square test showed that the difference is highly statistically significant, chi-square = 27.78 with 1 degree of freedom; $p < 0.0001$.

‡ The size of osteofoci in tumors generated by PC12-tet cells is significantly larger than the size of osteofoci in tumors generated by PC12 control cells. Statistical analysis was performed using Mann-Whitney rank sum test; 19 degrees of freedom; $t = 2.22$; $p < 0.05$. Values are the mean ± SEM.

§ The area of osteofoci in tumors generated by PC12-tet cells is significantly larger than the area of osteofoci in tumors generated by PC12 control cells. Statistical analysis was performed using Mann-Whitney rank sum test; 20 degrees of freedom; $t = 3.008$; $p < 0.01$. Values are the mean ± SEM.

pressed at sites of ongoing osteogenesis during limb development in the newborn mice.

Previous studies have shown that the tetranectin polypeptide and its mRNA have a wide tissue distribution (3, 10, 11, 12, 40). High tetranectin mRNA levels were found in several human normal tissues, i.e., placenta, heart, spleen, and lung, but little was detected in the liver or in the kidney (3, 40). Tetranectin is present in normal human plasma at a concentration of ~15 mg/l (14), and the plasma concentration of tetranectin is reduced in patients with various malignancies (22, 24). Neame et al. (30) found tetranectin to be an abundant component of shark cartilage. The role of tetranectin during bone formation in the mammals was explored in the present study. We found that tetranectin mRNA was expressed in newborn limbs and that the polypeptide was deposited in the newly formed woven bone. The *in vitro* experiments confirmed that the osteoblastic cells transcribe tetranectin mRNA.

The biosynthesis and biochemical processing of tetranectin has not been unequivocally determined. This has been hampered in part by the fact that little tetranectin production can be detected in cultured cell lines at either the protein or mRNA level. We were however able to show the biosynthesis of tetranectin using PC12-tet cells (Fig. 3). Previous studies using tetranectin polyclonal antiserum in Western blots have revealed an additional 95–100-kD immunoreactive band in some cultured cells and in leukocytes (6, 15). One suggestion was that the 95–100-kD band might be a tetranectin-related protein species. However, cDNA cloning and Northern blot analysis demonstrated a 1-kb mRNA of tetranectin (3, 40), strongly arguing against the existence of a precursor-product relationship. The data compiled in the present study shows that recombinant tetranectin produced by the PC12-tet cells is not incorporated into a 95–100-kD species, and therefore does not support the hypothesis that tetranectin complexes with another gene product.

During the process of osteogenesis, the expression of a number of noncollagenous proteins, such as alkaline phosphatase, osteopontin, osteonectin/SPARC, bone sialoprotein, osteocalcin, matrix protein Gla, and the small proteoglycans decorin and biglycan, varies spatially and temporally (2, 4, 5, 17, 20, 28, 35, 39). We now add to this list another protein present in the bone matrix, tetranectin. In the long bone of the newborn mouse, tetranectin immunoreactivity was found to be confined to the newly formed

woven bone in the periosteum and the primary spongiosum, suggesting that tetranectin is a rather late differentiation marker protein. No apparent tetranectin immunoreactivity was observed in the cartilage in the same sections. Likewise, Neame et al. (30) failed to detect tetranectin in mammalian cartilage. It should be noted however, that we have not yet examined tetranectin during embryogenesis and thus cannot exclude that tetranectin could be present in the cartilage of the developing fetus. Osteonectin/SPARC was found to be present in the hypertrophic zone of fetal mouse bone but absent at this location in the newborn mouse (35). Another aspect that remains to be explored is whether the gene expression pattern of tetranectin as assessed by *in situ* hybridization corresponds with the matrix deposition determined by immunohistochemical methods.

To specify the expression of tetranectin by osteoblasts as a function of time, we used an *in vitro* system (42) in which the differentiation of cultured bovine osteoblasts, proteins such as decorin and osteonectin/SPARC are induced early, while others such as bone sialoprotein are expressed at later stages (23). In this assay tetranectin was first detected 6 d after the switch to mineralization medium, at the stage when mineralizing nodules have started to form. At this stage the alkaline phosphatase mRNA expression was highest. The time course of tetranectin mRNA induction was similar to that of bone sialoprotein, which is thought to be a late differentiation marker (5, 17, 23), consistent with our immunolocalization results. In studies using primary cultures of normal diploid rat osteoblasts, levels of matrix Gla protein were present during the proliferative period and increased during the period of matrix formation (2, 31). The expression of osteocalcin, on the other hand, was initiated at the onset of mineralization (2, 31), which is comparable to the expression pattern of tetranectin observed in our *in vitro* assay system. Thus, the onset and pattern of tetranectin gene expression most closely resembles that of bone sialoprotein and osteocalcin, except its magnitude of enhancement. Since tetranectin mRNA levels remained high at the later stage of mineralization, tetranectin may not only be involved in the ongoing process of mineralization, but may have other functions in mature osteoblasts.

The mechanisms of bone formation have been explored using a variety of *in vivo* experimental systems (38). Some epithelial cell lines induce bone formation when implanted into connective tissue of nude mice (1, 43) and this was found

to correlate with ability of the epithelial cells to produce alkaline phosphatase-enriched matrix vesicles (7). During the process of bone induction at ectopic sites, mesenchymal cells migrate to the "implant" and differentiate into chondrocytes, which then synthesize and calcify their matrix. The calcified cartilage is vascularized and bone forms on the cartilage scaffold (38). Bone formation in general is attributable to a large number of bone morphogenetic proteins and bone-derived growth factors that may act in both a paracrine and autocrine fashion (17, 38). In the present study we used PC12 cells that induce and support osteogenesis after their injection into nude mice (our unpublished results), and we showed that the degree of bone induction was augmented by the overexpression of tetranectin. The role that tetranectin may play in osteogenesis is unknown, but it may relate to its ability to bind to plasminogen and to stimulate plasminogen activation by tissue-type plasminogen activator under certain conditions (14) and thereby contribute to the regulation of tissue formation and remodeling. Tetranectin binds to calcium and heparin (14) and thus could be an integral component of the bone matrix, which contains calcium and proteoglycans (17). In addition to tetranectin, other proteins such as thrombospondin and fibronectin are shared by the blood and the extracellular matrix (37). Finally, it is interesting to speculate that tetranectin interacts with growth factors, as has been shown with the other bone matrix proteins osteonectin/SPARC (32) and decorin (44).

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