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Human platelets contain and secrete osteonectin, a major protein of mineralized bone

(radioimmunoassay)

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ABSTRACT **We have developed a solid-phase competitive radioimmunoassay for human osteonectin, using a monoclonal antibody to bovine osteonectin. The assay is both specific and sensitive, being capable of measuring as little as 10 ng of osteonectin. Osteonectin measurements in parallel serum and plasma samples obtained from healthy individuals showed the plasma level to be 0.9 μg/ml, while that of serum was 3 times higher, 2.6 μg/ml. Radioimmunoassay of blood cells indicated that platelets contain osteonectin at 1.9 μg per 2×10^8 cells. Further, the protein is released after thrombin stimulation of these cells. Immunoblot analyses of washed pelleted human platelets resulted in the identification of a single immunoreactive species. The molecular weight of this immunoreactive species was identical to that obtained for purified bovine bone osteonectin. The isolation procedure developed for bovine bone osteonectin was applied to human platelets and bone. The individual steps of the isolation procedure yielded identical profiles of immunoreactive material for bone and platelet extracts. Results of reverse-phase high-pressure liquid chromatography of bone- and platelet-derived osteonectin are consistent with the conclusion that the two sources yield the identical protein.**

The noncollagenous protein fraction of cortical bone makes up approximately 2% of the total bone mass (1) and contains both proteins of bone cell origin and serum proteins that are concentrated in bone (2). Comparison of two-dimensional electrophoretograms of disodium ethylenediaminetetraacetate (Na_2EDTA) extracts of cortical bovine bone with electrophoretograms of bovine plasma have suggested the existence of at least 15 bone proteins of nonplasma origin (including actin) (3). One of these noncollagenous bone proteins, osteonectin, was isolated from fetal calf subperiostal bone and described by Termine *et al.* (4) and subsequently was isolated from adult bone by our laboratory (5). Osteonectin, a glycoprotein with a molecular weight of 29,000, exhibits high affinity for type I collagen and hydroxyapatite (6) and is the most potent inhibitor of hydroxyapatite crystal growth yet identified (5). This protein also possesses the ability to precipitate Ca^{2+} and PO_4^{3-} from stable solutions in the presence of collagen (6). These observations suggested that osteonectin may play an important role in the regulation of bone mineralization by binding hydroxyapatite to collagen. This view was further supported by immunochemical analyses indicating that osteonectin was present in bone and dentin but could not be found in nonmineralized tissue except for a small amount in fetal calf serum (6). More recently, however, the same laboratory reported the synthesis of an osteonectin-like protein by porcine periodontal ligament fibroblasts (7).

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Our laboratory has been able to isolate osteonectin under nondenaturing conditions (5) and to produce high-titer monoclonal antibodies to the native protein (8). Using these reagents, we developed a sensitive solid-phase radioimmunoassay to measure osteonectin in bone extracts and human serum samples. Osteonectin was found to be more abundant in adult bovine bone than in adult human bone and to be present at a greater concentration in human serum than in human plasma. The difference between serum and plasma osteonectin concentrations suggested that osteonectin was contained in and released from circulating blood cells during coagulation. This was investigated by using conventional immunochemical techniques.

MATERIALS AND METHODS

Osteonectin Isolation. Bovine femurs were obtained from a local slaughterhouse and human femurs from surgical amputations. The femurs were reduced to bone powder and demineralized according to previously described methods (9). Osteonectin was isolated from both species according to the method previously established by this laboratory for bovine osteonectin (5).

Human platelets were obtained from the American Red Cross of Vermont. Immediately upon preparation of platelet-rich plasma, an inhibitor cocktail was added (final inhibitor concentrations were 5 mM Na_2EDTA, 2 mM benzamidine, 1 mM ε-aminocaproic acid, soybean trypsin inhibitor at 0.02 mg/ml, 0.01 mM phenylmethylsulfonyl fluoride, heparin at 2 units/ml, 2.5 μM prostaglandin E_1, and 2 mM *N*-ethylmaleimide). Diisopropyl fluorophosphate was added to each platelet pack (equivalent to 1 unit of whole blood, ≈470 ml) to a final concentration of 1 μM after transport to the laboratory (≈10 min). Platelets were isolated and washed to remove plasma by the method previously described (10). Washed platelets were counted and then lysed with 1% Triton X-100 followed by sonication (W-375 Ultrasonics Inc., Plainview, NY; microtip setting 4.5, 50% duty cycle) for three sequential 1-min intervals. Platelet osteonectin was then isolated by the same procedure as bovine and human bone osteonectin, starting with the Sephadex G-100 fractionation step.

During isolation and subsequent storage, osteonectin becomes fragmented. Intact osteonectin can be resolved from fragments by using an Altex Ultrapore (Beckman, Fullerton, CA) RPSC (4.6 × 75 mm) high-pressure liquid chromatography (HPLC) column with a 0–60% (vol/vol) acetonitrile gradient over a 40-min time span. The purified intact osteonectin usually elutes at 38–39% acetonitrile with ≈23% recovery. Osteonectin concentrations were determined by using the extinction coefficient (A_{280} = 3.6 for a 1% solution) previously reported (5). Osteonectin purity before and after the HPLC step was assessed by sodium dodecyl sulfate/

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polyacrylamide gel electrophoresis ($NaDodSO_4$/PAGE) (5–15% acrylamide) according to the method of Laemmli (11).

Purified bovine osteonectin was labeled with carrier-free sodium [^{125}I]iodide (Amersham) by the chloramine-T method of Hunter and Greenwood (12). Osteonectin labeled in this manner (^{125}I-osteonectin) was >90% precipitable by trichloroacetic acid and was visualized as a single band after $NaDodSO_4$/PAGE and radioautography. The estimated specified activity for the ^{125}I-osteonectin is usually 55–65 μCi/μg (1 Ci = 37 GBq). Both the ^{125}I-osteonectin and the osteonectin used for standards in the radioimmunoassay were stored at −20°C in 50% (vol/vol) glycerol.

Radioimmunoassay for Osteonectin. A solid-phase competitive radioimmunoassay for osteonectin was developed. Immulon Removawells (Dynatech, Alexandria, VA) were coated with 0.1 ml of monoclonal anti-osteonectin (8) (100 μg/ml) in 0.5 M carbonate buffer, pH 10.0. After an overnight incubation at 4°C the antibody solution was removed and the wells were washed three times with 0.01 M sodium phosphate/0.15 M NaCl, pH 7.4 (PBS) and blocked with 0.2 ml of PBS with 2% bovine serum albumin. After blocking, the solution was removed and 0.1-ml portions of bovine osteonectin standards were added to each well. Osteonectin standards (0.01–30 μg/ml) were prepared by dilution in PBS/1% bovine serum albumin/1% Triton X-100, pH 7.4. An additional 0.1 ml, containing 10,000 cpm of ^{125}I-osteonectin, was added to each well. The wells were incubated at room temperature for 2 hr. After incubation the wells were washed three times with PBS/0.1% bovine serum albumin/0.1% Triton X-100 and placed in 12 × 75 mm glass tubes, and their radioactivities were measured. In addition to the wells containing osteonectin standards, wells with buffer were included for calculations of maximum ^{125}I-osteonectin binding. For osteonectin determinations in serum, plasma, platelet extracts, bone extracts, and other protein solutions, 0.1-ml samples were incubated with 0.1 ml of ^{125}I-osteonectin and treated in the same manner as the standards.

For serum and plasma studies, blood was collected from healthy donors into evacuated tubes containing either no anticoagulant or Na_2EDTA, sodium heparin, or sodium citrate. For coagulated plasma studies, Na_2EDTA anticoagulated blood was collected and centrifuged and the plasma was removed. The Na_2EDTA-containing plasma was split into three fractions; one remained anticoagulated and the other two were clotted, one by the addition of thrombin at 2 units/ml and the other by the addition of 20 mM $CaCl_2$ and 0.375 ml of thromboplastin (Sigma). Complete clot formation was obtained by incubation at 37°C for 10 min. Serum from the same individual was obtained for comparison purposes.

Human bone extracts and bovine bone extracts were from the first peak of the Sephadex G-100 gel filtration step in the osteonectin isolation procedure. For the preparation of platelet extracts, blood (6 parts) was collected into acid/citrate/dextrose (1 part) anticoagulant. Platelets were then isolated and washed by the method of Mustard *et al.* (13). The platelets were lysed in osteonectin assay buffer at a final concentration of 2×10^8 platelets per ml. For activated platelet extracts and released material 2×10^8 platelets were treated with thrombin at 1 unit/ml and incubated for 2 min with vigorous stirring (14). Aggregates were spun at 10,000 × *g*, the supernatant was harvested, and the aggregates were lysed with 1 ml of osteonectin assay buffer.

Other proteins screened in the osteonectin radioimmunoassay included coagulation factors II, V, IX, and X and protein C (6×10^{-8} to 6×10^2 μg/ml) isolated in this laboratory (15–19), bone Gla protein (osteocalcin; Gla indicating the presence of γ-carboxyglutamic acid) (6×10^{-8} to 6×10^2 μg/ml) isolated by the procedure of Price *et al.* (20), and fibronectin (0.001 to 10 μg/ml) (Collaborative Research, Waltham, MA).

Immunoblots of Laemmli Polyacrylamide Gels. Thrombin-activated and nonactivated platelet pellets were lysed and prepared for electrophoresis by the addition of $NaDodSO_4$ lysis buffer with heating for 5 min (21). These platelet extracts and purified bovine bone osteonectin were then subjected to electrophoresis (10–20% acrylamide) using the method of Laemmli (11). In some cases the proteins were transferred immediately to nitrocellulose, while in others the gel was stained first. For transfer with prior Coomassie blue staining, the conditions were the same, except that the gel was incubated in 25 mM Tris/glycine, pH 8.6/1% $NaDodSO_4$ for 1 hr. The transfer buffer was 25 mM Tris/glycine, pH 8.6/20% (vol/vol) methanol (22). For transfer without staining, the transfer was performed at 110 V for 2 hr in a buffer recycling system to maintain a constant temperature of 10°C. The transfer buffer was 20 mM Tris/glycine, pH 8.6, with a final $NaDodSO_4$ concentration of 0.01%. Immediately after transfer the nitrocellulose sheet, (0.45-μm pores, Schleicher & Schuell) was placed in a blocking buffer of 1% bovine serum albumin in PBS for 1 hr. After blocking, the nitrocellulose sheet was incubated in a solution of monoclonal anti-osteonectin in 1% bovine serum albumin/PBS (5.5 mg/30 ml). After a 1-hr incubation with the primary antibody blots were subjected to three sequential 20-min washes: PBS, PBS/0.01% Nonidet P-40, and finally PBS again. After washing, blots were incubated for 1 hr with 5×10^6 cpm of goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA), labeled with sodium [^{125}I]iodide (Amersham) by the method of Hunter and Greenwood (12). After incubation with the second antibody the nitrocellulose sheets were washed as before, dried, and placed in an x-ray cassette with Du Pont Kronex Lightning Plus intensifying screens and exposed to Kodax XRA5 film for 48 hr at −70°C.

RESULTS

The solid-phase osteonectin radioimmunoassay generated a standard curve with sensitivity from 0.01 to 2 μg with an apparent K_d of 5.3×10^{-9} M (Fig. 1 *Upper*). Dilutions of serum and plasma, from 10^0 to 10^{-1}, were parallel with the bovine osteonectin standards diluted in buffer (Fig. 1 *Upper*). Intra- and interassay coefficients of variation [CV = $(SD/\bar{X}) \times 100\%$], calculated from repeated assays of a normal serum pool, were 5.9% and 10.6%, respectively. Dilutions of gel-filtered (Sephadex G-100) extracts of human and bovine bone resulted in superimposable assay curves that were completely parallel with the bovine osteonectin standard curve (data not shown). Fig. 1 *Lower* illustrates the lack of cross-reactivity of the monoclonal anti-osteonectin for blood coagulation factors X, V, IX, and II and protein C over the range tested (6×10^{-8} to 6×10^2 μg/ml). Bone Gla protein at 60 μg/ml exhibited a slight amount of cross-reactivity, which is insignificant since this concentration is 10,000 times the circulating concentration of bone Gla protein. Fibronectin did not cross-react with anti-osteonectin. However, as demonstrated in Fig. 1 *Upper* the osteonectin concentration of serum, 2.6 μg/ml, differs markedly from that obtained for plasma, 0.9 μg/ml. This represents a 2.9-fold difference in osteonectin concentration, which cannot be accounted for by assay variation.

Recovery of osteonectin from serum and plasma was studied by adding bovine osteonectin at 3, 2, 1, and 0.5 times their respective concentrations. The recoveries of added osteonectin from serum and plasma were 90% and 100%, respectively, eliminating the notion that a component of plasma, absent in serum, could mask osteonectin from the assay. To analyze the production of an osteonectin-like substance during the activation of the blood coagulation

proenzymes, studies of clotted plasma were performed. Plasma was harvested from Na_2EDTA-anticoagulated blood and split into three aliquots. Two plasma aliquots were clotted, one by the addition of thrombin (2 units/ml) and the other by Ca^{2+} and thromboplastin. The remaining plasma aliquot was left anticoagulated. Dilution curves of the two clotted plasma supernatants were superimposable on the anticoagulated plasma dilution curve, and all were parallel with the bovine standard curve. Serum osteonectin levels from the same individual, prepared by clotting whole blood, were again 2.5 times greater than the plasma levels. The above experiments indicate that the immunoreactive species is not contributed by the activation of coagulation components.

The primary cellular event that occurs in association with coagulation is the activation and aggregation of platelets. Platelets, when activated, release their α-granule constituents as well as products associated with the dense bodies and lysosomes (23). Extracts of isolated platelets were assayed for immunoreactivity; they were found to contain an osteonectin-like substance at 1.9 μg per 2×10^8 platelets. The immunoreactivity measured in these platelet extracts represents approximately 2.85 μg of antigen per ml of blood, based upon a normal platelet count of 3×10^8 platelets per ml. The total immunoreactivity measured in the supernatant of 2×10^8 thrombin-stimulated platelets (1.5 μg) was similar to that found in the lysates from 2×10^8 unstimulated platelets (1.2 μg). This indicated that an immunoreactive species, quantitatively equivalent to the measured antigen, is released from platelets upon activation.

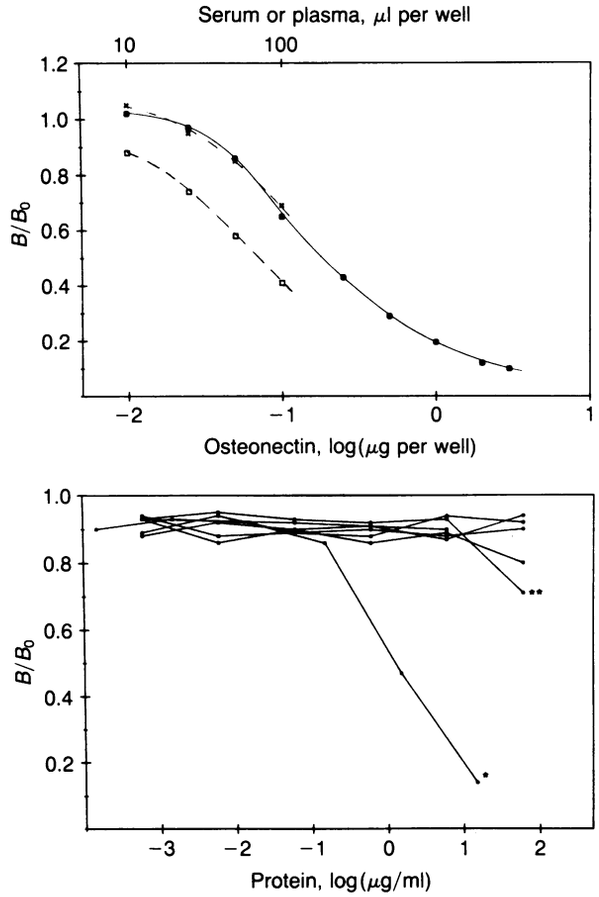

FIG. 1. Osteonectin radioimmunoassay. B, ^{125}I-osteonectin bound; B_0, amount bound in the absence of competitor. (*Upper*) Dilutions of purified bovine bone osteonectin displaced ^{125}I-labeled antigen over the range of 0.01–2 μg per well (0.1 ml per well) (■). Human serum (□) and plasma (×) over the range 10–100 μl per well gave displacement curves parallel to the curve obtained with purified bovine bone standards. (*Lower*) Cross-reactivity was examined by comparing dilutions of osteonectin (*) to bone Gla protein (**) and other calcium-binding proteins (coagulation factors II, V, IX, and X and protein C).

Immunochemical visualization of the platelet immunoreactive species and its comparison to human and bovine bone osteonectin was accomplished by $NaDodSO_4$/PAGE and immunoblot analyses. An autoradiograph of an immunoblot comparing an extract from 1.5×10^8 isolated human platelets (lane 4 in Fig. 2) with purified bovine osteonectin at 5, 1, and 0.2 μg (lanes 1, 2, and 3 in Fig. 2) illustrates a band of immunoreactivity at an apparent molecular weight indistinguishable from that of bovine osteonectin. Platelet activation effected by thrombin stimulation resulted in the release of platelet-associated osteonectin and hence a loss of immunoreactivity (lanes 7 and 8 in Fig. 2) when compared to a lysate from the same number of unactivated pelleted platelets (lanes 9 and 10 in Fig. 2). The bovine bone osteonectin standards for the thrombin stimulation experiment are shown in lanes 5 and 6. There was no apparent change in the stained pattern of a comparison gel (lanes 13 and 14 vs. 11 and 12). This verifies that the immunoreactivity is released from platelets when they are thrombin activated and not merely associated with the platelet surface.

The platelet immunoreactive species appeared to be electrophoretically identical to osteonectin. Platelet osteonectin was therefore isolated according to the bone osteonectin isolation procedure, monitored by using the immunoassay. Fractionation of the platelet osteonectin antigen completely mimicked the osteonectin isolations from human and bovine bone. The starting material, from four platelet packs, contained 0.596 mg of immunoreactive material, on the basis of the osteonectin radioimmunoassay. After DEAE-Sephadex A25 (Pharmacia) chromatography the protein concentration as measured by absorbance at 280 nm was equivalent to 1.1 mg of osteonectin. However, lane 4 of the $NaDodSO_4$/PAGE pictured in Fig. 3 *Upper*, when compared to the autoradiograph in Fig. 3 *Lower*, indicates that, although the osteonectin present is identical to that of bone, not all of the protein present in the platelet preparation is osteonectin. To further purify the platelet, human bone, and bovine bone osteonectin preparations, HPLC was per-

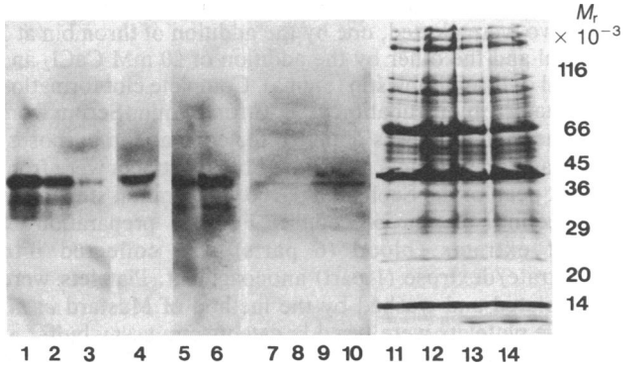

FIG. 2. Platelet osteonectin documented by $NaDodSO_4$/PAGE and immunoblot analyses of 2-mercaptoethanol-reduced samples. Monoclonal anti-osteonectin, ^{125}I-labeled antibody to mouse IgG, and autoradiography were used to detect purified osteonectin standards at 5 μg (lane 1), 1 μg (lane 2), and 0.2 μg (lane 3). In the same experiment platelet osteonectin was visualized in an extract from 1.5×10^8 platelets (lane 4). In a subsequent experiment osteonectin standards (lanes 5 and 6) were compared to unactivated platelet extracts (lanes 13 and 14, Coomassie blue; lanes 9 and 10, autoradiography). Extracts of thrombin-activated platelet pellets are analyzed by Coomassie blue in lanes 11 and 12 and by autoradiography in lanes 7 and 8.

formed. The chromatograms were similar for each of the three sources, with the osteonectin peak being eluted between 38% and 40% acetonitrile.

For osteonectin derived from bone the HPLC step is a useful means of obtaining intact osteonectin free of smaller molecular weight fragments. The platelet osteonectin, however, contained a number of protein bands, at various molecular weights, that could not be removed by using the HPLC protocol. The relative purity of the various osteonectin preparations is illustrated in Fig. 3 *Upper*. Lane 2 represents purified bovine bone osteonectin and derived fragments. Lane 3 represents the same preparation after removal of fragments by HPLC. Lanes 4 and 5 correspond to osteonectin isolated from human platelets before and after HPLC, respectively. Human bone osteonectin is represented in lane 6, before HPLC, and in lane 9 is purified human bone osteonectin after HPLC.

Although the bone osteonectin preparations contain no higher molecular weight bands as judged by Coomassie blue staining, an autoradiographic immunoblot (Fig. 3 *Lower*) of the same gel after staining showed a band of reactivity at a molecular weight of approximately 70,000 (lanes 2, 3, and 6). The purified bovine and human bone osteonectins also contain a slightly lower molecular weight band, visualized by immunoreactivity, that was not detectable by Coomassie blue staining (lanes 3 and 9, respectively), but the majority of the immunoreactivity is at the molecular weight of intact osteonectin. However, both the pre- and post-HPLC platelet osteonectin preparations, which contain a number of components by Coomassie blue staining, display only one distinct band of immunoreactivity, located at the molecular weight of osteonectin (lanes 3 and 4, respectively). In addition, a slight amount of immunoreactivity can be seen in the platelet osteonectin preparation; this material corresponds to the high molecular weight components in the bone preparations.

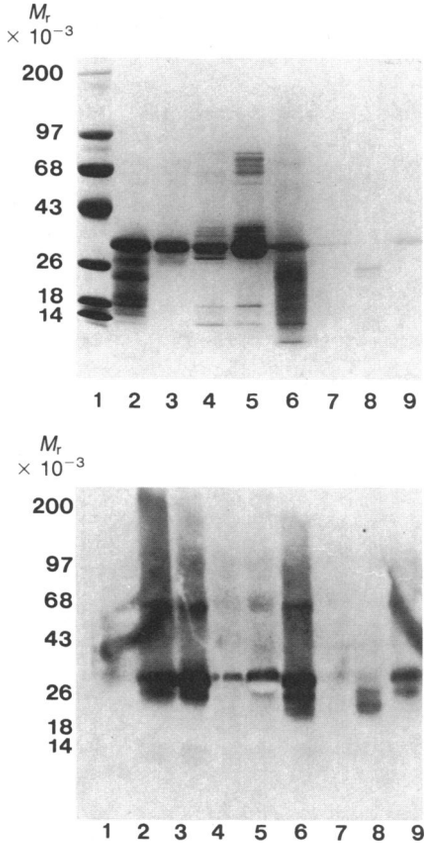

FIG. 3. Coomassie blue and immunoblot analyses of osteonectin isolated from bovine bone, human bone, and human platelets. (*Upper*) Coomassie blue-stained $NaDodSO_4$/PAGE analysis of nonreduced osteonectin samples obtained by the standard isolation procedure from bovine bone (lane 2), human platelets (lane 4), and human bone (lane 6). These osteonectin preparations were further purified by HPLC and are represented in lanes 3, 5, and 9, respectively. Lanes 7 and 8 are components of the human bone osteonectin preparation (fragments) removed by HPLC. Lane 1 is molecular weight standards. (*Lower*) Immunoblot analysis of the gel shown in *Upper*.

DISCUSSION

The nondenaturing isolation procedure developed for bovine osteonectin by Romberg *et al.* (5) was modified by using reverse-phase HPLC, resulting in the isolation of highly purified osteonectin from human bone. The availability of purified osteonectin and monoclonal anti-bovine osteonectin antibodies, which cross-react with the human protein, has enabled us to develop a human osteonectin radioimmunoassay that is both sensitive and specific. The parallel determinations of osteonectin concentrations in serum and plasma samples from the same individuals demonstrated that the serum concentration was approximately 3 times greater than that of plasma. Experiments utilizing the radioimmunoassay indicated that platelets contain osteonectin, which can be released after thrombin stimulation. This additional store of osteonectin appears to represent the major source of serum osteonectin. We have confirmed the identification of the immunoreactive species as osteonectin by immunoblot analyses. Whole platelet lysates were compared to purified bovine bone osteonectin by using murine monoclonal antibodies to bovine osteonectin. Platelet osteonectin has also been isolated from freshly prepared platelet-rich plasma, utilizing the procedure developed for the purification of the bone protein. Platelet osteonectin antigen behaved at each purification step in a manner identical to bone protein, further verifying its identity with the bone species.

The high affinity of osteonectin for calcium ions and collagen, coupled with its release from thrombin-activated platelets, provides a basis for comparison to other proteins also released from platelets during thrombin activation: fibronectin (24), thrombospondin (25, 26), fibrinogen (27–29), and von Willebrand factor (30). These proteins are collectively implicated as adhesion proteins due to their ability to bind to cell surfaces, resulting in cellular aggregation or adherence both *in vitro* and *in vivo* (31–36). These similarities are further supported by the adherence capabilities of the cell types found to contain osteonectin: calvarial bone cells (37), fibroblasts (7), and platelets. Collectively, these similarities suggest that osteonectin may serve to support a mechanism by which bone cells are anchored.

The function of osteonectin in platelets is unknown; however, its presence and high concentration would appear to render it a poor indicator of osteoblast function when measured in serum or plasma. However, the radioimmunoassay developed for osteonectin should play an important role in elucidating its function in tissues by determining concentrations *in situ* as well as amounts produced by clonal bone cell lines.

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