**Human Platelet Osteonectin: Release, Surface Expression, and Partial Characterization**

By Robert J. Kelm, Jr., and Kenneth G. Mann

Our laboratory has previously shown that osteonectin, an abundant noncollagenous bone protein, is contained in and secreted from human platelets. In this study, the distribution of osteonectin both in the supernatant and on the platelet surface after activation was measured by fluid-phase and solid-phase radioimmunoassay, respectively. Total cellular osteonectin was determined by RIA of guanidinium chloride extracted platelets and ranged from 0.65 to 2.2 pg/10^8 platelets or 135,000 to 457,000 molecules/platelet. Platelets treated with varying concentrations of collagen and thrombin released osteonectin in a dose-dependent fashion. Approximately 61% of the total platelet osteonectin was secreted at saturating concentrations of collagen and thrombin. A small fraction of platelet osteonectin is expressed on the surface of platelets in an activation-specific manner as evidenced by the specific and saturable binding of ^[125]I-anti-osteonectin monoclonal antibody, IIIA3A8, to thrombin-activated platelets. Based on a nonlinear least squares regression analysis of the antibody binding, 2,200 IIIA3A8 molecules, or 0.8% of the total platelet osteonectin, is expressed on the platelet surface on activation. Platelet osteonectin was purified from the supernatant of thrombin-activated platelets by immunofinity chromatography. Western blotting of proteins secreted by washed, thrombin-stimulated platelets with IIIA3A8 indicated that the osteonectin molecule released from the platelet is a single chain polypeptide. Comparison of immunopurified platelet osteonectin with isolated bone bone osteonectin and isolated human bone osteonectin by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that platelet osteonectin has a greater apparent molecular weight than bone osteonectin. The NH2-terminal sequence of immunopurified platelet osteonectin was obtained by automated Edman degradation and is identical to the sequence of human bone osteonectin derived from the cDNA of SaOS-2 cells. Collectively, these data suggest that platelet osteonectin is structurally distinct from bone osteonectin in a region of the molecule at a distance from the NH2-termínus.

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OSTEONECTIN, a 29,000 molecular weight phosphoglycoprotein, is one of the major protein constituents of the mineral matrix of bone. Osteonectin was originally isolated from the demineralized bone matrix of fetal bovine bone by Termine et al., and later from adult bovine and human bone. It accounts for approximately 23% of the total noncollagenous protein in compact bone. Among osteonectin's better characterized properties are its ability to bind Ca^2+ , collagen, and hydroxyapatite and to inhibit hydroxyapatite crystal growth. Purification of osteonectin from fetal porcine bone has also been reported. However, unlike bovine bone osteonectin, porcine osteonectin is reported to have no affinity for either native or denatured collagen, nor does it selectively bind hydroxyapatite. The complete amino acid sequence of bovine osteonectin from normal fetal bovine bone cells and human osteonectin from the osteosarcoma cell line SaOS-2 has been derived from cDNA technology. The NH2-terminal sequence of bovine, porcine, and human osteonectin is almost completely identical to two acidic secreted glycoproteins (SPARC: secreted protein acidic and rich in cysteine) from mouse parietal endoderm cells and 43K protein from bovine aortic endothelial cells. The amino acid sequence of human SPARC obtained from a placenta cDNA library is identical to human osteonectin. Polyclonal antibodies raised toward bovine osteonectin crossreact with both mouse SPARC and bovine 43K protein, suggesting that the three proteins share common epitopes and are members of a family of similarly structured proteins. To date, the function of osteonectin, as it pertains to bone or other metabolic processes, has not been clearly established.

In a previous study from this laboratory, human platelets were identified as containing an antigen that crossreacts with an anti-osteonectin monoclonal antibody (MoAb). The concentration of this immunoreactive species was measured by radioimmunoassay (RIA) of detergent solubilized platelets and found to be 1.9 µg/2 x 10^8 platelets or about 200,000 molecules/platelet. This antigen was also shown to be released by platelets when the cells were activated with 1.0 U/mL thrombin. We also reported that partially purified platelet osteonectin and purified bone osteonectin apparently comigrate when analyzed by Western blotting of two-dimensional polyacrylamide gels. These observations led to the conclusion that osteonectin, at the time a presumed bone-specific protein, was also an abundant platelet protein. These observations, coupled with the fact that osteonectin has been localized in a variety of cell and tissue types by immunocytotchemical staining or in situ hybridization including osteosarcoma, periodontal ligament fibroblasts, and human decidua and carcinoma, indicate that the biologic function of osteonectin is not restricted to bone. Because one of the primary physiologic functions of the platelet is to secrete proteins and other metabolites involved in the regulation of hemostasis, in particular, those that modulate adherence of platelets to each other and to the vascular endothelium.

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Submitted August 21, 1989; accepted November 8, 1989.

Supported by NIA Project 3 of Grant POI AG04875 B. Lawrence Riggs, Principle Investigator, Mayo Clinic, Rochester, MN.

Presented in preliminary form at the Joint Meeting of the International Conference on Calcium Regulating Hormones and the American Society of Bone and Mineral Research, September 9-14, 1989, Montreal, Quebec, Canada.

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0006-4971/90/7505-0021$3.00/0

Blood, Vol 75, No 5 (March 1), 1990: pp 1105-1113

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lrium, it is conceivable that osteonectin, an extracellular matrix-associated protein, could play a role in this process. This hypothesis is supported by a recent study which reported that purified platelet and bone osteonectin bind to thrombospordin, an adhesive platelet glycoprotein. In this study we use both solution-phase and solid-phase RIA's to quantitate the distribution of osteonectin in the supernatant and on the platelet surface after platelet activation. We studied the secretion of osteonectin from human platelets after stimulation by known platelet agonists, collagen and thrombin, and report that although a substantial proportion of the total osteonectin is released, a significant fraction remains associated with the activated platelet. We also report the specific binding of a radiolabeled MoAb directed against osteonectin to washed, thrombin-activated platelets, suggesting that at least a fraction of the nonreleasable osteonectin is expressed on the surface of the platelet in an activation-specific manner. Analysis of osteonectin, immunoaffinity purified from the supernatant of aggregated platelets, by SDS-polyacrylamide gel electrophoresis indicates that platelet osteonectin has a higher apparent molecular weight than bone-derived osteonectin, implying that the two proteins are chemically distinguishable.

MATERIALS AND METHODS

Materials. Heparin, prostaglandin E1 (PGE1), apyrase, trisodium citrate, dextrose, calcium chloride, magnesium chloride, (2-Hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), benzamidine HCl, RIA grade bovine serum albumin, fatty acid free bovine serum albumin, and cyagen bromide activated Sepharose-4B were purchased from Sigma (St Louis, MO). Trizma base, sodium chloride, and sodium phosphate were from Baker (Rochester, NY). Apiezon and N-butyl phthalate were from Fisher (Springfield, NJ). EDTA was from EM Science (Fort Washington, PA). Carrier-free Na125I iodide was obtained from Amersham (Arlington Heights, IL). Guanidinium chloride was purchased from Heico (Delaware Water Gap, PA). ACA-34 gel filtration resin was from Pharmacia LKB (Piscataway, NJ). DEAE-cellulose was from Whatman (Kent, England). Cibacron Blue F3GA was from Pierce (Rockford, IL). Ammonium sulfate was obtained from Swarts-Mann (Cleveland, OH). Platelet factor-4 RIA kit was obtained from Abbott (North Chicago, IL). Bovine femurs were obtained from a local slaughterhouse. Human bone was acquired as specimens that would have been otherwise disposed of after surgical excision.

Protein isolation. Prothrombin was purified from human plasma as described by Mann. Thrombin was purified as described by Lundblad et al. Anti-osteonectin immunoglobulin (IgG), IIIA4, was isolated from murine ascites fluid using a combination of gel filtration and ion exchange chromatography at 22°C. Ascites fluid was passed over a 2.5 × 90 cm ACA-34 column equilibrated in 0.02 mol/L Tris, 0.15 mol/L NaCl pH 7.4. The peak containing the IgG was diluted twofold to 0.01 mol/L Tris, 0.075 mol/L NaCl, and applied to a 1.5 × 10 cm DEAE-cellulose column equilibrated in the same buffer. The IgG present in the flow-through was further purified using a 1.0 × 5.0 cm Cibacron Blue F3GA column in 0.001 mol/L Tris, 0.075 mol/L NaCl pH 7.4. The bound IgG was eluted via a step gradient, 0.01 mol/L Tris, 0.3 mol/L NaCl, and precipitated by making the eluant 70% ammonium sulfate. The precipitate was collected after centrifugation at 10,000 rpm for 20 minutes. The pellet was resuspended in 0.001 mol/L NaPO4, 0.15 mol/L NaCl pH 7.4, and dialyzed versus the same buffer. The purity of the IgG was assessed via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli. The concentration of the IgG was assessed by optical density measurement using an extinction coefficient of E1%280,0.1cm = 14, correcting for Rayleigh scattering at 320 nm using the equation described previously.

Bovine bone osteonectin was isolated from 0.5 mol/L EDTA, 0.05 mol/L benzamidine HCl extracts of finely milled bovine bone femurs as previously described. Human bone osteonectin and human platelet osteonectin were purified using immunoadfinity chromatography. Anti-osteonectin IgG, IIIA4, 20 mg, was coupled to 4.0 mL wet volume CNBr-activated Sepharose 4B according to the method of Parikh et al. Human platelet osteonectin was prepared from the supernatant of washed, thrombin-activated platelets. Blood, a total of 480 mL, was drawn from informed, consenting, nonsmoking, aspirin-free adults through 19-gauge butterfly needles into 0.156 mol/L citrate, 0.1 mol/L dextrose, 5.0 × 10−6 mol/L PGE1. Platelets were washed according to the method of Mustard et al and counted using a Coulter apparatus (Coulter Electronics LTD, Hialeah, FL). Platelets, 4.5 × 109, were resuspended in 0.02 mol/L Tris, 0.15 mol/L NaCl pH 7.6, and activated by the addition of 2.5 U/mL thrombin. The activated, aggregated platelets were removed by centrifugation at 10,000 rpm for 30 minutes.

The supernatant from the activated platelets was applied to the IIIA4 affinity column at 4°C. The column was washed and the platelet supernatant was incubated with the antibody-conjugated resin for 16 hours at 4°C. After the overnight incubation, the column was washed with 0.02 mol/L Tris, 0.15 mol/L NaCl pH 7.6 until an absorbance at 280 nm of 0.01 was obtained. The column was then washed with 0.02 mol/L Tris, 1.0 mol/L NaCl pH 7.6 to baseline absorbance. Human platelet osteonectin was subsequently eluted with 3.0 mol/L NaSCN, 0.02 mol/L Tris, 0.15 mol/L NaCl pH 7.6. The osteonectin contained in the 3.0 mol/L NaSCN peak was dialyzed extensively versus 0.05 mol/L NH4HCO3 and lyophilized.

Whole bone extract of finely milled human bone was prepared as described for bovine bone. The 0.5 mol/L EDTA extract was desalted on a Sephadex G-25 column at 4°C, and the flow-through peak containing the noncollagenous bone proteins was dialyzed extensively versus 0.02 mol/L Tris, 0.15 mol/L NaCl pH 7.6 at 4°C. Insoluble material resulting from dialysis was removed by centrifugation at 10,000 rpm for 30 minutes. The supernatant was applied to the IIIA4 affinity column equilibrated in 0.02 mol/L Tris, 0.15 mol/L NaCl pH 7.6 at 4°C at a flow rate of 0.5 mL/min. The column was washed and eluted as described for human platelet osteonectin preparation. The purity of the isolated human osteonectin from bone and platelet was assessed by SDS-PAGE. The proteins were visualized by staining with either Coomassie Blue R250 or silver nitrate. Protein concentration of osteonectin was obtained by optical density measurement using an extinction coefficient E1%280,0.1cm of 3.6.

Release experiments. Release studies on washed human platelets were performed as described by Kaplan. Platelets were washed and isolated from whole blood drawn into 0.156 mol/L citrate, 0.1 mol/L dextrose in the absence of PGE1. Washed platelets, at a concentration of 2 × 1011/mL, in a volume of 0.45 mL 0.35% Tyrode's albumin containing 0.005 mol/L HEPES, 0.005 mol/L CaCl2, 0.001 mol/L MgCl2, pH 7.4, were activated by the addition of agonist, 50 μL, diluted in 0.01 mol/L NaPO4, 0.15 mol/L NaCl pH 7.4. Collagen (Chrono-Log, Haverton, PA) was diluted from a stock solution of 1.0 mg/mL in 1.0 mmol/L HCl. The concentration of collagen was calculated using a molecular weight of 300,000 for trimeric collagen. Aggregation of the stirred platelet suspensions was followed using a Chrono-Log dual channel aggregometer that was blanked against platelet-free Tyrode's albumin. After 3 minutes, the aggregometer cuvette was placed on ice. In the case of thrombin stimulation immediately before placing the platelets on ice, hirudin
was added to the suspension to a final concentration $5.0 \times 10^{-4}$ mol/L to inactivate the thrombin. The platelet supernatant was obtained by centrifugation of the activated platelets in an Eppendorf microfuge (Brinkmann Instruments, Westbury, NY) for 3 minutes at 10,000 rpm. The supernatant was removed and stored at $-20^\circ$C.

Frozen and thawed platelet supernatants were analyzed for osteonectin by the radioimmunoassay described by Stenner et al. Platelet factor-4 RIAs were also performed on the same platelet supernatants according to the manufacturer's specifications. Total platelet antigen was determined by an RIA of 4.0 mol/L guanidine chloride, 0.005 mol/L benzamidine extracts of unactivated platelet suspensions following extensive dialysis against 0.01 mol/L NaPO$_4$, 0.15 mol/L NaCl pH 7.4. Percent release was calculated from the ratio of released antigen to total antigen.

Platelet binding studies. Anti-osteonectin MoAb, IIIA$_A$, and bovine bone osteonectin were radioiodinated with carrier-free Na$_{125}$Iodide via the chloramine T method. The radiolabeled proteins were greater than 90% precipitable with 10% trichloroacetic acid (TCA) and were visualized as a single band after SDS-PAGE and radioautography. The specific activities of bone osteonectin and IIIA$_A$ were 6,300 and 2,200 cpm/ng, respectively. For binding experiments, platelets were isolated from whole blood drawn into 0.156 mol/L citrate, 0.1 mol/L dextrose containing $5.0 \times 10^{-6}$ mol/L PGE$_2$. Washed platelets at a concentration of $4 \times 10^{10}$/mL were added to a Tyrode's albumin solution containing varying concentrations of the labeled ligand in 12 x 75 mm plastic tubes, in the presence or absence of a 100-fold excess of unlabelled ligand to a final volume of 0.25 mL. If platelet activation was desired, 0.5 U thrombin in 10 mL 0.01 mol/L NaPO$_4$, 0.15 mol/L NaCl pH 7.4 was added to the solution containing the labeled ligand immediately before the addition of the platelet suspension. Total incubation time was 30 minutes. This interval was determined by time course experiments in which saturation of platelet binding sites was achieved by 30 minutes. Duplicate aliquots were removed and layered over 0.5 mL 1 part apiezon A:9 parts N-buty1 phthalate in a 1.5-mL Eppendorf tube (Brinkmann Instruments). Platelets were pelleted in an Eppendorf microfuge for 2 minutes at 10,000 rpm. The tips of the tubes were amputated and counted in a Beckman gamma-counter. Specific binding of IIIA$_A$ was calculated by subtracting nonspecific binding due to entrapment from total binding. The number of antibody molecules bound to the platelet at saturation was determined by fitting the experimental data using nonlinear least squares regression analysis to a second order polynomial equation, TWWONL, described by Cleland.

Immunoblotting of osteonectin. The supernatant from thrombin-activated platelets along with the elution peaks from the immunoaffinity column were analyzed for platelet osteonectin by Western blotting of a Coomassie Blue-stained 3% to 12% Laemmli SDS-polyacrylamide gel. The gels were soaked in 0.048 mol/L Tris, 0.019 mol/L glycine, 20% methanol, 1% SDS pH 8.6 for 1 hour. The proteins were transferred to nitrocellulose (0.45 $\mu$m pores, Hoefer) using a Gelman semi-dry blotting apparatus for 2 hours, 150 mA, at 22$^\circ$C. The transfer buffer was 0.048 mol/L Tris, 0.039 mol/L glycine, 0.0013 mol/L SDS, 20% methanol. After transfer, the nitrocellulose sheet was placed in a blocking buffer of 1% bovine serum albumin (BSA), 0.02 mol/L Tris, 0.15 mol/L NaCl pH 7.4 for 2 hours. After blocking, the nitrocellulose was incubated with radiolabeled anti-osteonectin IgG, 13 x 10$^{6}$ cpm [125]IIIIA$_A$/50 mL 1% BSA, 0.02 mol/L Tris, 0.15 mol/L NaCl pH 7.4 for 2 hours. After incubation with the antibody, the nitrocellulose was washed twice with 0.02 mol/L Tris, 0.15 mol/L NaCl, 0.05% Tween 20 pH 7.4 for 20 minutes each. The nitrocellulose was then washed with 0.02 mol/L Tris, 0.15 mol/L NaCl pH 7.4 for 20 minutes and air-dried. The nitrocellulose was placed in an x-ray cassette with intensifying screens and exposed to Kodak XRA5 film (Eastman Kodak, Rochester, NY) for 5 hours at $-70^\circ$C.

Protein sequencing. The NH$_2$-terminal sequence of human platelet osteonectin was obtained by automated Edman degradation using an Applied Biosystems 475A Protein Sequencing System (Applied Biosystems, Foster City, CA). The sample was prepared by desalting immunoaffinity purified human platelet osteonectin on a GPC-100 HPLC gel filtration column in 0.05 mol/L ammonium formate. The osteonectin, 100 pmoles, was lyophilized and resuspended in 10% acetic acid for application to the sequencer.

RESULTS

Initial measurements of total platelet osteonectin were determined by RIA of washed, nonactivated platelets after extraction with 4.0 mol/L guanidine chloride. In five experiments conducted with a different suspension of washed platelets from normal donors, the measured immunoreactive osteonectin in guanidine chloride extracted resting platelets was 0.65 to 2.52 µg/10$^8$ platelets (153,000 to 457,000 molecules/platelet). The range of values measured for total platelet osteonectin likely reflects individual donor variability. To account for donor variability in terms of the total amount of antigen contained within the platelet at a given time, the measured amount of osteonectin secreted from agonist activated platelets is expressed as a percentage of the total osteonectin measured in guanidine chloride extracts of unactivated platelets from the same donor. This permitted the comparison of the release of osteonectin in platelets obtained from different donors. Initial assays of the supernatants of platelets obtained from a single donor indicated that on stimulation with varying concentrations of the platelet agonists, thrombin and collagen, osteonectin is released from the platelets in a dose-dependent manner. The secretion of platelet factor-4, an α-granule protein that has been previously demonstrated to be released by platelets stimulated with thrombin or collagen, paralleled the release of osteonectin, suggesting that at least a fraction of the total platelet osteonectin is most likely stored in the α-granules of platelets and is secreted in a contemporaneous manner with platelet factor-4.

Compilation of the platelet osteonectin secretion data obtained from several experiments using different preparations of washed platelets indicated that the release of osteonectin from platelets stimulated with either thrombin or collagen is agonist concentration-dependent (Fig 1A and B). In addition, at agonist concentrations of 1.5 nmol/L and 15 nmol/L for thrombin and collagen respectively, the release of osteonectin approaches a maximum. The amount of osteonectin secreted by the platelet at saturating concentrations of thrombin and collagen is on average 61% of the total platelet osteonectin (Fig 1). This value is consistent regardless of the total amount of osteonectin initially present in a particular donor's platelets (Table 1). Based on these results it is apparent that the platelet does not release its entire store of osteonectin when activated.

The results of the release experiments indicated that a significant fraction of the total platelet osteonectin remains associated with the platelet after activation. In an attempt to address this issue in terms of the location of this nonreleasable fraction, the surface of the activated platelet was
for entrapment of the radiolabeled ligand. The data indicated that bone osteonectin does not bind to thrombin activated platelets because addition of a competitor, 100-fold molar excess of unlabeled bone osteonectin, did not displace the radiolabeled osteonectin associated with the platelet pellet. From these observations, assuming that the radiiodination procedure did not structurally alter the osteonectin molecule, the conclusion that one may draw is that exogenously added bone osteonectin does not bind to the surface of activated platelets. Thus, assessment of the interaction of bone osteonectin with the platelet would not be useful in determining the location of the nonreleasable fraction of platelet osteonectin. For this reason, the issue of the location of the nonreleasable fraction of platelet osteonectin was approached using a methodology that made use of a MoAb specific for both bone and platelet osteonectin.

The MoAb, IIIA3A8, which was used to measure the release of osteonectin from activated platelets, also served as a marker for endogenous osteonectin expression on the surface of platelets. The interaction of the anti-osteonectin MoAb with both resting and thrombin-activated platelets was investigated using a radioligand binding assay. Specific and saturable binding of the antibody to platelets required the intentional activation of the platelet (Fig 2). Platelets were incubated with varying concentrations of radiolabeled IIIA3A8 in the presence and absence of a 100-fold molar excess of unlabeled IIIA3A8 to account for nonspecific binding due to entrapment of the radiolabeled ligand. To account for possible antibody-Fc receptor interaction, antibody binding to platelets was also measured after preincubation of the antibody with bovine bone osteonectin at concentrations sufficient to saturate all antigen combining sites (a 100-fold molar excess of bovine bone osteonectin relative to antibody concentration). Specific binding of radiolabeled

Fig 1. Dose response curves of osteonectin release from washed platelets activated with thrombin (A) or collagen (B). Washed human platelets (0.5 mL), at 4 x 10^8/mL, were incubated, with stirring, with varying concentrations of thrombin and collagen for 3 minutes at 37°C. Platelets were pelleted and the supernatants were assayed for osteonectin by RIA. Percent release was calculated from the ratio of secreted osteonectin to total osteonectin in a guanidinium chloride lysate of 2 x 10^8 unactivated platelets. Each point represents the mean ± 1 SEM for three individual experiments.

Table 1. Relative Distribution of Osteonectin in Resting Platelets and in the Supernatant of Platelets Activated With Saturating Concentrations of Collagen and Thrombin From Different Donors

<table>
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<th>Donor</th>
<th>Released</th>
<th>Total Molecules/Platelet</th>
<th>Molecules/Platelet (Collagen)*</th>
<th>Molecules/Platelet (Thrombin)†</th>
<th>Mean % Released</th>
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<td>1</td>
<td>457,000</td>
<td>280,000</td>
<td>251,000</td>
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<tr>
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*Collagen concentration, 33 nmol/L.
†Thrombin concentration, 2.7 nmol/L.

Fig 2. Specific binding of [125I] anti-osteonectin MoAb, IIIA3A8, to resting (open symbols) and thrombin-activated (closed symbols) platelets. Washed platelets, 1 x 10^8 in 0.250 mL, from two different donors (circles and triangles) were incubated with varying concentrations of [125I]-III A3A8 in the presence or absence of competitor for 30 minutes at 22°C. An aliquot from each reaction mixture was layered over oil and the platelets were sedimented. Platelet-associated radioactivity was quantitated using a gamma-counter. Specific binding was calculated by subtracting nonspecific binding from total binding.

examined for its potential to bind osteonectin. Initially, the interaction of radiolabeled bovine bone osteonectin with the surface of the activated platelet was assessed using a radioligand binding assay. Washed, thrombin-activated platelets were incubated with varying concentrations of radiolabeled bovine bone osteonectin. Parallel reaction mixtures containing both the radioactive ligand and a 100-fold molar excess of unlabeled bovine bone osteonectin were included to control
anti-osteonectin to platelets was calculated by subtracting nonspecific binding from total binding. As demonstrated in Fig 2, IIIA₃A₈ binds preferentially to thrombin-activated platelets. These data support the conclusion that the expression of immunoreactive osteonectin on the surface of the platelet is an activation specific phenomenon.

The quantitation of the number of antibody binding sites was achieved by fitting the experimental data using nonlinear least squares regression analysis to a theoretical second order polynomial equation in which the asymptote estimates the number of molecules bound at saturation. These data indicate that there are 2,200 antibody binding sites/platelet. Assuming a 1:1 stoichiometry for antigen/antibody binding, at least 2,200 molecules may be expressed on the surface of an activated platelet. Table 2 compares the number of IIIA₃A₈ binding sites with other known MoAbs that bind preferentially to activated platelets.

Characterization of the relationship of platelet osteonectin to its counterpart derived from demineralized bone matrix required the purification of osteonectin contained within the platelet. Isolation of human platelet osteonectin using conventional techniques developed for the isolation of bovine bone osteonectin did not produce sufficient quantities of high quality platelet osteonectin for chemical analysis. For this reason, an alternative methodology was used to purify human platelet (as well as bone) osteonectin. The supernatant from thrombin-activated platelets was passed over a column in which an anti-osteonectin MoAb, IIIA₃A₈, was coupled to Sepharose 4B (Fig 3A). Laemmli SDS-polyacrylamide gels (Fig 3B, upper panel) and the corresponding immunoblots (Fig 3B, lower panel) of the various protein peaks off the column, run under both nonreduced (Fig 3B, A through E) and reduced (Fig 3B, F through J) conditions, demonstrate the specificity of the column for binding osteonectin out of platelet releasate derived from 4.5 × 10⁹ washed platelets. The arrows indicate the position of the platelet osteonectin in the starting material (Fig 3B, lanes A and F) and in 3.0 mol/L NaSCN elution peak (Fig 3B, lanes D and I). Immunoblotting of the starting material, platelet releasate, shows that osteonectin is released from the platelet as a single polypeptide chain. This is consistent with a previous observation from this laboratory in which one band of immunoreactivity was seen on a Western blot of Triton X-100 solubilized unactivated platelets. The apparent absence of osteonectin in the flow through peak (Fig 3B, lanes B and G) demonstrates that the column selectively removed osteonectin from the supernatant. Treatment of the column with 1.0 mol/L NaCl resulted in the elution of a protein that migrated with an apparent molecular weight of 450,000 under nonreduced conditions (Fig 3B, lane C) and 150,000 under reduced conditions (Fig 3B, lane H). The apparent molecular weight of this species under both reduced and nonreduced conditions is consistent with that of thrombospondin, an a-granule protein that has been reported to selectively bind osteonectin. However, in contrast to Clezardin et al., who have also reported the purification of human platelet osteonectin, the apparent molecular weight of the platelet osteonectin eluted from the column with 3.0 mol/L NaSCN (Fig 3B, lanes D and I) is slightly larger than that of purified bovine bone osteonectin (Fig 3B, lanes E and J). This molecular weight difference is not due to species variability since immunopurified human bone osteonectin does not comigrate with immunopurified human platelet osteonectin. The observed apparent molecular weight difference between human platelet osteonectin and human bone osteonectin when analyzed by SDS-PAGE analysis suggested that there might be a chemical difference between the protein isolated from bone matrix and that which is purified from the supernatant of activated platelets. Analysis of the NH₂-terminus of the immunosolated platelet osteonectin by automated Edman degradation (Table 3) showed that the NH₂-terminus of osteonectin isolated from platelets gives a result identical to that which is predicted from osteonectin cDNA derived from SaOS-2 cells. Thus, the difference between the two proteins likely resides in a region at a distance from the NH₂-terminus.

DISCUSSION

The concentration of osteonectin in guanidinium chloride extracts of five different platelet preparations ranged from 0.65 to 2.2 μg/10⁹ platelets or 135,000 to 457,000 molecules/platelet. The amount of osteonectin detected in 1% Triton X-100 solubilized platelets previously reported by our laboratory, 0.95 μg/10⁹ platelets or 197,000 molecules/platelet is well within the range measured in guanidinium chloride extracts. Osteonectin, like other well-characterized secreted platelet proteins, in particular platelet factor-4, β-thromboglobulin, fibrinogen, fibronectin, thrombospondin and von Willebrand factor, is released from human platelets on activation. In this study, both thrombin and collagen were shown to stimulate the release of osteonectin from platelets in a dose-dependent manner. The release of osteonectin parallels the release of platelet factor-4, suggesting that at least part of the total platelet osteonectin is contained within the platelet a-ganule. Approximately 61% of the total platelet osteonectin is released on activation.

The observation that release of osteonectin was incomplete with respect to the total amount of detectable platelet antigen suggested to us that a significant fraction of the total platelet osteonectin remained associated with the platelet after activation and release. We examined the nature of this association by determining whether or not osteonectin was expressed on the surface of the platelet after activation. Although we observed no binding of exogenously added bone osteonectin to platelets which may have resulted from

<table>
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<th>Antibody</th>
<th>Agonist</th>
<th>Binding Sites/Platelet</th>
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<tr>
<td>IIIA₃A₈</td>
<td>Thrombin</td>
<td>2,200</td>
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<tr>
<td>KC4*</td>
<td>Thrombin</td>
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<td>ADP/epi</td>
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<tr>
<td></td>
<td>Thrombin</td>
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<tr>
<td>2.28§</td>
<td>Thrombin</td>
<td>12,600</td>
</tr>
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*Data from Hsu-Lin et al.†
†Data from McEver and Martin.‡
‡Data from Shattil et al.¶
§Data from Nieunenhuis et al.
competition by released platelet osteonectin, we have shown that an anti-osteonectin MoAb, IIIA, binds specifically and saturably to thrombin-activated platelets but not to resting platelets. This indicated that osteonectin is expressed on the surface of platelets in an activation-specific manner. The proportion of osteonectin expressed on the activated platelet, 2,200 molecules, is approximately 1.0% of the total osteonectin found in platelets. Estimation of the equilibrium
weight glycoprotein. In a later study, McEver and Martin\textsuperscript{34} also demonstrated the secretion and activation-dependent association of a MoAb, S12, with platelets. This antibody was also shown to crossreact with an antigen of 140,000 molecular weight. In subsequent studies both KC4\textsuperscript{25} and S12\textsuperscript{26} were found to recognize the identical protein. This protein called either PADGEM (platelet activation dependent granule to external membrane protein) or GMP-140 (granule membrane protein) is localized in the \( \alpha \)-granule membrane of resting platelets, but is exposed on the surface of the platelet on activation. In another study, Nieuwenhuis et al\textsuperscript{37} reported the activation-specific binding of an MoAb, 2.28, to platelets. The antigen recognized by this antibody was a 53,000 molecular weight protein. Unlike PADGEM, this protein is located in the lysosomal membrane of unactivated platelets. Several other platelet-specific antibodies, including PAC-1\textsuperscript{38} and 7E3,\textsuperscript{39} have been used by investigators to study the expression of the platelet membrane receptor protein complex GPIIb/IIIa. These antibodies are specific markers for activation-dependent changes in the GPIIb/IIIa complex epitope expression and bind to the platelet in the absence of platelet secretion. Although the abundance of binding sites for IIIA3A8 is small relative to other platelet membrane-specific antibodies (Table 2), it is conceivable that IIIA3A8 could potentially serve as a useful and specific surface marker for platelet activation.

The phenomenon of activation-specific expression of an endogenous platelet protein is not without precedent. A variety of adhesive proteins found in the platelet are known to bind to the platelet on activation. These include thrombospondin,\textsuperscript{40} von Willebrand factor,\textsuperscript{41,42} and fibrinogen,\textsuperscript{43} which bind to specific platelet membrane receptors, in particular, the GPIIb/IIIa complex in the case of fibrinogen and von Willebrand factor. These molecules are believed to mediate platelet aggregation and adhesion to the vascular endothelium at the onset of a thrombotic event. Because osteonectin is known to bind to collagen, it is conceivable that osteonectin may play a regulatory role in mediating adhesion of the platelet to the vascular endothelium after activation.

Purification of released platelet osteonectin was accomplished using immunoaffinity chromatography. The MoAb, IIIA3A8, used to describe both the release and surface expression of platelet osteonectin, was covalently coupled to Sepharose-4B and specifically removed a single species of osteonectin from a platelet supernatant, as assessed by SDS-PAGE and immunoblotting (Fig 3). Initial elution of the resin with 1.0 mol/L NaCl resulted in the purification of a protein that migrated at an apparent molecular weight of 450,000 nonreduced and 150,000 reduced. The molecular size of this protein in SDS-polyacrylamide gels is consistent with that reported for thrombospondin, a platelet protein previously reported to bind osteonectin.\textsuperscript{18} Thrombospondin is contained within the \( \alpha \)-granule and is known to bind to platelets via a specific membrane receptor\textsuperscript{44} not related to the GPIIb/IIIa complex. Thrombospondin has also been recently demonstrated to be synthesized and secreted by the osteoblast or bone-forming cell.\textsuperscript{45} Our data, with respect to the immunoaffinity purification of platelet osteonectin, suggests that osteonectin and thrombospondin are either re-

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### Table 3. The NH\(_2\)-Terminal Sequence of Immunopurified Human Platelet Osteonectin

<table>
<thead>
<tr>
<th>Cycle No.</th>
<th>Amino Acid</th>
<th>pmol</th>
<th>Repetitive Yield</th>
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<td></td>
</tr>
<tr>
<td>2</td>
<td>P</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Q</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Q</td>
<td>20</td>
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<tr>
<td>5</td>
<td>E</td>
<td>30</td>
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<tr>
<td>6</td>
<td>A</td>
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<td>93</td>
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</table>
leased from the platelet as a complex, or alternatively, form a complex in solution once the two proteins are secreted from the platelet. It is therefore conceivable that the osteonectin expressed on the surface of the activated platelet is actually bound to platelet surface-associated thrombospondin. Further experiments are needed to characterize the nature of the osteonectin-thrombospondin interaction both in solution and possibly at the cell surface of both platelets and bone cells. These studies will be important in terms of understanding the function of osteonectin in regard to its role in cell-cell and cell-extracellular matrix interactions.

The platelet osteonectin eluted from the affinity column by 3.0 mol/L NaSCN has a mobility on SDS-polyacrylamide gels that is clearly slower than that of bone osteonectin (Fig 3). The relative molecular weight difference between bone and platelet osteonectin is approximately 3,000 and suggests that the protein derived from the platelet is chemically different from that derived from bone matrix. This difference was not noted in our initial studies, probably because of the loss of resolving power in the second dimension of two-dimensional gel electrophoresis, or potentially from an altered electrophoretic mobility due to the presence of Triton X-100. The NH$_2$-terminal sequence data for platelet osteonectin reported in this study indicates the human bone and human platelet osteonectin have identical amino-termini. This suggests that the difference exists in a region of the molecule that is remote from the NH$_2$-terminus and likely results from posttranslational processing.

The molecular weight of bovine bone osteonectin isolated under non-denaturing conditions has been determined by sedimentation equilibrium analysis. The molecular weight of the molecule in both 0.15 mol/L NaCl and 6.0 mol/L guanidinium chloride was shown to be 29,000. However, the molecular weight calculated from the derived amino acid sequence of a human osteonectin cDNA from Saos-2 osteosarcoma cells not including carbohydrate is 32,700. Because only one gene for osteonectin has been characterized, it is possible that the osteonectin isolated from bone matrix represents a proteolyzed product derived from the intact molecule. Incorporation of osteonectin into the bone matrix after secretion from the osteoblast may require the proteolytic processing of osteonectin. The osteonectin isolated from the supernatant of washed, thrombin-activated platelets may represent the intact molecule, or alternatively, a unique posttranslationally processed form of osteonectin. Resolution of the structural difference between bone and platelet osteonectin may lead to a more in-depth understanding of the biologic function of this protein.

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[Image 0x0 to 581x788]
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