Agonist-activated αvβ3 on Platelets and Lymphocytes Binds to the Matrix Protein Osteopontin*

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The phosphorylated acidic glycoprotein osteopontin is present in the extracellular matrix of atherosclerotic plaques and the wall of injured but not normal arteries. To determine if osteopontin could serve as a substrate for platelet adhesion, we measured the adherence of resting and agonist-stimulated human platelets to immobilized recombinant human osteopontin. Agonist-stimulated but not resting platelets bound to osteopontin by a process that was mediated primarily by αvβ3. αvβ3-mediated adherence occurred at physiologic concentrations of calcium and was inhibited by an αvβ3-selective cyclic peptide. Assays using phorbol myristate acetate-stimulated transfected B lymphocytes expressing both αvβ3 and αIIbβ3 confirmed that activated αvβ3 not activated αIIbβ3 was responsible for the cellular adherence we measured. These studies indicate that αvβ3 can reside on the cell surface in an inactive state and can be converted to a ligand binding conformation by cellular agonists. Moreover, they suggest that platelet adherence to osteopontin mediated by activated αvβ3 could play a role in anchoring platelets to disrupted atherosclerotic plaques and the walls of injured arteries. By inhibiting αvβ3 function, it may be possible to inhibit platelet-mediated vascular occlusion with a minimal effect on primary hemostasis.

The final event in a variety of cardiovascular diseases is often arterial occlusion by a platelet-rich thrombus. Members of the integrin superfamily of adhesion molecules play a key role in this pathologic process by anchoring platelets to the exposed subendothelium of damaged arteries and by mediating platelet aggregation. The integrin, αIIbβ3, mediates platelet aggregation when platelet stimulation converts it from a resting to a ligand-binding conformation (1). Platelets contain a second β3 integrin, αvβ3, but whether αvβ3 plays a role in platelet function is not known. However, a monoclonal antibody that binds to both αIIbβ3 and αvβ3 has been shown in clinical trials to have additional efficacy relative to compounds that bind only to αIIbβ3 by preventing the reocclusion that often occurs months after PTCA (2–4).

Formation of an occlusive thrombus or a normal hemostatic platelet plug is initiated when platelets adhere to newly exposed components of the subendothelial extracellular matrix of diseased or damaged blood vessels. The matrix components assumed to function as substrates for platelet adherence include collagen, fibronectin, and von Willebrand’s factor because platelets contain receptors for each of these proteins and adhere to these proteins in vitro (2). Nevertheless, the substrates that actually mediate platelet adherence to disrupted atherosclerotic plaques are not known. Osteopontin is an acidic phosphorylated glycoprotein secreted by a number of cells including osteocytes, osteoclasts, macrophages, and smooth muscle cells (5–7). Although not present in the walls of normal arteries, osteopontin is widely distributed throughout the matrix of calcified plaques in arteries involved by atherosclerosis (8–10). Studies in vitro suggest that osteopontin may be involved in the formation of the neointima characteristic of the atherosclerotic process by serving as a substrate for αv-integrin-mediated smooth muscle and endothelial cell migration (8, 11). Because it is likely that osteopontin is exposed to circulating blood by the plaque disruption that precedes acute coronary artery occlusion and results from PTCA, we examined the possibility that osteopontin could serve as an adhesive substrate for platelets. We found that activated but not resting platelets adhere to osteopontin and that their adherence is mediated by an activated conformation of αvβ3.

EXPERIMENTAL PROCEDURES

Synthesis of Recombinant Human Osteopontin—Recombinant human osteopontin was synthesized as a histidine-tagged fusion protein using the pEET system (Novagen). A cDNA for human osteopontin was inserted into the plasmid pET16b, and recombinant protein was synthesized as insoluble inclusion bodies in Escherichia coli BL21(DE3)pLysS. Following lysis of the pelletted bacteria in 20 mM Tris-HCl buffer, pH 7.9, containing 0.5 mM NaCl, 1 mg/ml lysozyme, and 0.1% Triton X-100, osteopontin was solubilized using 6 M guanidine HCl and isolated by metal chelate affinity chromatography on a Ni²⁺–nitrilo-triacetic acid resin (His-Bind Resin, Novagen). Recombinant osteopontin was eluted from the resin using 20 mM Tris-HCl buffer, pH 7.9, containing 0.5 mM NaCl and 500 mM imidazole and renatured by dialysis against phosphate-buffered saline, pH 7.4. 0.1% SDS-7.5% polyacrylamide gel electrophoresis of the renatured protein revealed a single band with an apparent molecular weight of 58,000. The mass of the recombinant protein as determined by electrospray mass spectroscopy was 35,518, consistent with the calculated mass of the full-length osteopontin amino acid backbone (12) plus the polyhistidine tag and Factor Xa cleavage site contributed by pET16b.

Measurement of Platelet Adherence to Osteopontin and Fibrinogen—96-well flat bottom microtiter plates (Immulon 2, Dynatech) were coated with 5 μg/ml recombinant osteopontin, purified human fibrinogen, or bovine serum albumin, each dissolved in 50 mM NaHCO₃ buffer, pH 8.6, containing 150 mM NaCl. Unoccupied protein binding sites on the wells were blocked with 5 mg/ml bovine serum albumin dissolved in the same buffer. Human platelets were isolated from blood anticoagulated with 0.1 volume of 3.8% sodium citrate by gel filtration using a 4
Platelet Adherence to Osteopontin and Fibrinogen—To determine if osteopontin could serve as a substrate for platelet adherence, we used a solid phase assay to measure the adherence of gel filtered human platelets to either purified recombinant human osteopontin or purified human fibrinogen (13), a known adhesive ligand for platelets (16). In the presence of Mg++, there was substantial adherence of unstimulated platelets to fibrinogen but little adherence to osteopontin (Fig. 1A). Platelet stimulation with 10 μM ADP resulted in a dramatic increase in the number of platelets adherent to osteopontin, as well as a smaller increase in the number of platelets adherent to fibrinogen. Inspection of the assay plates by light microscopy confirmed these results and revealed that ADP stimulation resulted in both platelet adherence and spreading on the fibrinogen and osteopontin-coated surfaces (Fig. 1B). Similar results were observed when the platelets were stimulated by 20 μM epinephrine, 0.1 unit/ml thrombin, or 200 ng/ml PMA, and the presence of 25 μM indomethacin had no effect on the adherence of ADP-stimulated platelets. The addition of EDTA prevented platelet adherence to either substrate. To verify that activated platelets adhere to native osteopontin, as well as to recombinant protein, assays were repeated using purified osteopontin isolated from human urine (uropon tin). We found no difference in the ability of uropontin and recombinant osteopontin to support platelet adherence (data not shown). Thus, stimulated but not unstimulated human platelets are able to use immobilized osteopontin as an adhesive substrate.

Identification of the Receptor Responsible for Platelet Adherence to Osteopontin—To identify the receptor on activated platelets that mediates platelet adherence to osteopontin, we repeated the adherence assays in the presence of monoclonal antibodies against αIbβ3 and against αvβ3, the only αv-containing integrin present in platelets (Fig. 2). Although agonist-mediated platelet adherence is generally mediated by αIbβ3 (1), platelet adherence to osteopontin was consistently inhibited by 84–93% by the αvβ3-specific mAb LM609 (19) and 96–99% by the β3-integrin-specific mAb 7E3 (20). In contrast, saturating concentrations of the αIbβ3-selective mAbs A2A9 (17) and 10E5 (18) inhibited platelet adherence to osteopontin by only 30–40%, perhaps because these antibodies also cross-react with αvβ3 to some extent (15), whereas an antibody specific for α5β1 (mAb 16, Becton Dickinson) was not inhibitory.

RESULTS AND DISCUSSION

Platelet Adherence to Osteopontin and Fibrinogen—To determine if osteopontin could serve as a substrate for platelet adherence, we used a solid phase assay to measure the adherence of gel filtered human platelets to either purified recombinant human osteopontin or purified human fibrinogen (13), a known adhesive ligand for platelets (16). In the presence of Mg++, there was substantial adherence of unstimulated platelets to fibrinogen but little adherence to osteopontin (Fig. 1A). Platelet stimulation with 10 μM ADP resulted in a dramatic increase in the number of platelets adherent to osteopontin, as well as a smaller increase in the number of platelets adherent to fibrinogen. Inspection of the assay plates by light microscopy confirmed these results and revealed that ADP stimulation resulted in both platelet adherence and spreading on the fibrinogen and osteopontin-coated surfaces (Fig. 1B). Similar results were observed when the platelets were stimulated by 20 μM epinephrine, 0.1 unit/ml thrombin, or 200 ng/ml PMA, and the presence of 25 μM indomethacin had no effect on the adherence of ADP-stimulated platelets. The addition of EDTA prevented platelet adherence to either substrate. To verify that activated platelets adhere to native osteopontin, as well as to recombinant protein, assays were repeated using purified osteopontin isolated from human urine (uropon tin). We found no difference in the ability of uropontin and recombinant osteopontin to support platelet adherence (data not shown). Thus, stimulated but not unstimulated human platelets are able to use immobilized osteopontin as an adhesive substrate.

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Activated Platelet and Lymphocyte αβ3 Binds to Osteopontin

FIG. 3. Adherence of human B lymphocytes expressing αIIbβ3 and/or αβ3 to immobilized osteopontin and fibrinogen. The adherence of stable B cell lines expressing either αIIbβ3 and αβ3 (αIIbβ3) or αβ3 alone (GM1500) to osteopontin and fibrinogen in the presence or the absence of 200 ng/ml PMA was measured as described under “Experimental Procedures.” Identity of the receptor mediating lymphocyte adherence was determined by performing the assay in the presence of A2A9 or LM609. The data shown are the means and standard errors of triplicate determinations.

(draft text not shown). Inhibition by the tetrapeptide RGDS was nearly complete, consistent with platelet adherence to osteopontin being an integrin-mediated process. Conversely, there was nearly complete inhibition of platelet adherence to fibrinogen by A2A9, 10E5, and 7E3 and only minimal inhibition by LM609 (data not shown). Thus, these data indicate that whereas platelet adherence to fibrinogen is mediated by αIIbβ3, the receptor primarily mediating platelet adherence to osteopontin is αβ3. The data also indicate that the ability of platelet αβ3 to recognize osteopontin requires platelet stimulation.

There are 50-fold (21) to 500-fold (22) fewer copies of αβ3 compared with αIIbβ3 on the platelet surface. Moreover, αβ3 is generally considered to reside on the surface of most cells in a constitutively active state (23). To verify that agonist-stimulated platelet adherence to osteopontin is mediated by αβ3 rather than αIIbβ3, we used a B lymphocyte model of platelet integrin function. B lymphocytes constitutively express αβ3, but express αIIbβ3 after transfection (15, 24). Following exposure to PMA, only the transfected cells expressing αIIbβ3 bind soluble fibrinogen (24) or adhere to immobilized fibrinogen (15). We found little adherence of transfected lymphocytes to either osteopontin or fibrinogen in the absence of PMA stimulation (Fig. 3). Following PMA stimulation, lymphocyte adherence to osteopontin and fibrinogen increased by 13.6- and 8.1-fold, respectively. Nevertheless, whereas adherence to fibrinogen was inhibited by the mAb A2A9, indicating it was mediated by αIIbβ3, adherence to osteopontin was inhibited by the mAb LM609, indicating it was mediated by αβ3. Next, we measured the adherence of the parental line GM1500 that expresses αβ3 but not αIIbβ3 to both substrates (Fig. 3). As anticipated, the parental cells did not adhere to fibrinogen; however, their adherence to osteopontin was identical to that of the transfected cells. These data indicate that αβ3 on lymphocytes, like αβ3 on platelets, resides on the cell surface in an inactive state and confirm that agonist-generated intracellular signals can induce αβ3 binding to osteopontin.

Because the αβ3 on activated platelets and lymphocytes interacts with osteopontin, whereas αIIbβ3 on these cells interacts with fibrinogen, the α subunit of β3 integrins regulates their ligand binding specificity. The heavy chains of α and αIIb exhibit 38% overall homology, but homology increases to 57% when their amino-terminal halves containing their putative calcium binding domains are compared (25). Recent studies of αIIb and α concluded that the amino-terminal one-third of each protein is involved in ligand recognition (26), a conclusion consistent with peptide cross-linking studies implicating two sites in α encompassing amino acids 139–349 (27) and a site in αIIb that contains amino acids 294–314 (28). A major difference between the proximal end of the putative α ligand binding domain and the corresponding region of αIIb is the presence of a stretch of 10 additional amino acids in αIIb (Gly148–Glu157) (25), perhaps accounting for the different ligand preference of the integrins containing these α subunits.

Role of Divalent Cations in Platelet Adherence to Osteopontin—αβ3-mediated cell adhesion to osteopontin occurs in the presence of Mg2+, although Ca2+ can support osteopontin binding to purified αβ3 (29). To verify that Ca2+ can support platelet adherence to osteopontin, we measured the adherence of ADP-stimulated platelets suspended in buffer containing either Ca2+ or Mg2+ (Fig. 4). In the presence of EDTA, there was no adherence of ADP-stimulated platelets to osteopontin. However, both Ca2+ and Mg2+ supported platelet adherence to osteopontin in a concentration-dependent manner. At cation concentrations up to 1 mM, there was little difference in the ability of Ca2+ and Mg2+ to support platelet adherence; although at higher concentrations, the ability of Ca2+ to support adherence declined relative to that of Mg2+. Nevertheless, at a physiologically relevant concentration of 1 mM, Ca2+ supported platelet adherence to osteopontin nearly as well as Mg2+. Moreover, adherence in either Ca2+ or Mg2+-containing buffer was inhibited by LM609, indicating that it was mediated by αβ3 regardless of the divalent cation present (data not shown).

The divalent cation dependence of platelet αβ3 deviates significantly from that of αβ3 in certain cell lines where Ca2+ does not support cell adherence to osteopontin and can even be inhibitory (29). It has been reported that the αβ3-mediated adherence of 293 cells to osteopontin occurs in media containing Ca2+ but only when αβ1 is exposed to an activating monoclonal antibody, suggesting that the affinity state of αβ1 determines its ability to interact with Ca2+ (30). We found that only αβ3 on activated platelets mediates adherence to os-
Osteopontin

Fibrinogen

FIG. 5. Inhibition of ADP-stimulated platelet adherence to osteopontin and fibrinogen by the cyclic αvβ3-selective peptide XJ735. ADP-stimulated platelet adherence to osteopontin and fibrinogen was measured in the presence of increasing concentrations of XJ735 as described in the legend to Fig. 1 and under "Experimental Procedures." The data were normalized to 100% adherence in the absence of XJ735 to facilitate comparisons. The data are the means of triplicate determinations.

interaction of platelet αvβ3 with osteopontin. A potential advantage of this approach is that it may be less prone to impair hemostasis than current therapies. Indeed, peptides that bind to αvβ3 have been shown to be effective in animal models for restenosis (33), and we have shown that XJ735 has significant, αIIbβ3-independent, in vivo antithrombotic efficacy but does not increase bleeding times in dogs and swine. Thus, αvβ3-mediated platelet adherence to osteopontin could be involved in the pathogenesis of acute arterial occlusion, and inhibitors of this integrin may be useful pharmaceutical agents for treating arterial thrombotic disorders.

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