Polymeric Osteopontin Employs Integrin α9β1 as a Receptor and Attracts Neutrophils by Presenting a de Novo Binding Site

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Osteopontin (OPN) is a cytokine and ligand for multiple members of the integrin family. OPN undergoes the in vivo polymerization catalyzed by cross-linking enzyme transglutaminase 2, which consequently increases the bioactivity through enhanced interaction with integrins. The integrin α9β1, highly expressed on neutrophils, binds to the sequence SVVYGLR only after intact OPN is cleaved by thrombin. The SVVYGLR sequence appears to be cryptic in intact OPN because α9β1 does not recognize intact OPN. Because transglutaminase 2-catalyzed polymers change their physical and chemical properties, we hypothesized that the SVVYGLR site might also be exposed on polymeric OPN. As expected, α9β1 turned into a receptor for polymeric OPN, a result obtained by cell adhesion and migration assays with α9-transfected cells and by detection of direct binding of recombinant soluble α9β1 with colorimetry and surface plasmon resonance analysis. Because the N-terminal fragment of thrombin-cleaved OPN, a ligand for α9β1, has been reported to attract neutrophils, we next examined migration of neutrophils to polymeric OPN using time-lapse microscopy. Polymeric OPN showed potent neutrophil chemotactic activity, which was clearly inhibited by anti-α9β1 antibody. Unexpectedly, mutagenesis studies showed that α9β1 bound to polymeric OPN independently of the SVVYGLR sequence, and further, SVVYGLR sequence of polymeric OPN was cryptic because SVVYGLR-specific antibody did not recognize polymeric OPN. These results demonstrate that polymerization of OPN generates a novel α9β1-binding site and that the interaction of this site with the α9β1 integrin is critical to the neutrophil chemotaxis induced by polymeric OPN.

Acidic phosphorylated secreted glycoprotein osteopontin (OPN), known as a cytokine, has multiple functions, including roles in tissue remodeling, fibrosis, mineralization, immunomodulation, inflammation, and tumor metastasis (1–3). OPN is also an integrin ligand. At least nine integrins can function as OPN receptors. α5β1, αβ81, αβ81, αβ3, αβ5 (1), and αβ6 (4) recognize the linear tripeptide RGD, and α9β1, α4β1, and α4β7 recognize the sequence, SVVYGLR (5), adjacent to RGD but only after OPN has been cleaved by the protease, thrombin (Fig. 1).

The overlap of receptors for OPN does not necessarily mean that these integrins play redundant roles in cellular responses to OPN because the patterns of integrin expression and utilization vary widely among cell types. In addition, interactions of different integrins with a single ligand can exert distinct effects on cell behavior in a single cell type. For example, we have previously reported that signals by ligation of αβ3, αβ6, or α9β1 to a single ligand, tenascin-C, differently affected cell adhesion, spreading, and proliferation of the colon cancer cell line, SW480 (6). Furthermore, intact OPN or thrombin- or matrix metalloproteinase-cleaved OPN interact with distinct subsets of integrins and exhibit distinct effects on cell behavior (4, 7, 8).

Collectively, some of the functional diversity of OPN could be attributed to this multiplicity of receptors and responses. We have recently shown that polymerization of OPN results in enhanced biological activity (9). We thus set out to determine whether polymerized OPN exerts its effects through unique interactions with integrins.

OPN is polymerized by transglutaminase 2 (TG2, EC 2.3.2.13) (10) that catalyzes formation of isopeptide cross-links between glutamine and lysine residues in substrate proteins (11) including OPN. Polymeric OPN has been identified in vivo in bone (12) and calcified aorta (13). We have previously reported that upon polymerization, OPN displays increased integrin binding accompanied by enhanced cell adhesion, spreading, migration, and focal contact formation (9). However, very little is known about how polymeric OPN induces its biological effects.

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Integrin α9β1, highly expressed on neutrophils (14), does not act as a receptor for intact OPN but does bind to an N-terminal fragment of OPN (nOPN) that is generated by thrombin cleavage (15) through the new C-terminal sequence, SVVYGLR. Protein polymerization can expose otherwise cryptic domains (16), so we hypothesized that the SVVYGLR site might be exposed upon polymerization and serve as a binding site for α9β1. In the present study, we demonstrate that α9β1 is indeed a receptor for polymeric OPN and that neutrophil migration induced by polymeric OPN is largely mediated by this interaction. However, mutational analysis and antibody studies demonstrate that this interaction does not involve the SVVYGLR site, suggesting the presence of de novo binding site in polymeric OPN.

**EXPERIMENTAL PROCEDURES**

**Cells, Antibodies, and Reagents**—Mock- and α9-transfected human colon cancer cell line SW480 (17) and monoclonal antibody (mAb) specific for integrin α9β1 (Y9A2) (18) were provided by Dr. Dean Sheppard (University of California, San Francisco (UCSF), San Francisco, CA). FreeStyle 293F was obtained from Invitrogen. Anti-human integrin mAbs specific for α5β1 (JBS5), αvβ5 (P1F6), and β2 subunit (MEM48) were from Chemicon. Anti-OPN mAb 34E3 was from IBL (Takasaki, Japan). Mouse anti-V5 antibody was from Invitrogen. Horse-radish peroxidase-conjugated antibodies against mouse IgG and chicken IgY were from SouthernBiotech and Bethyl Laboratories, respectively. TG2 was prepared and provided by Dr. Yuji Saito (Tokyo Institute of Technology, Yokohama, Japan). Recombinant tenascin-C fragment of OPN (nOPN) that is generated by thrombin cleavage site (16), so we hypothesized that the SVVYGLR site might be exposed upon polymerization and serve as a binding site for α9β1. In the present study, we demonstrate that α9β1 is indeed a receptor for polymeric OPN and that neutrophil migration induced by polymeric OPN is largely mediated by this interaction. However, mutational analysis and antibody studies demonstrate that this interaction does not involve the SVVYGLR site, suggesting the presence of de novo binding site in polymeric OPN.

**Recombinant OPN Proteins**—As described previously (5), OPN proteins were expressed as glutathione S-transferase fusion protein in Escherichia coli with pGEX6P plasmid, and affinity-purified then cleaved from glutathione S-transferase with PreScission protease (GE Healthcare). Human polymeric OPN was generated by incubation with guinea pig TG2 (9) and purified by anion-exchange chromatography (Applied Biosystems).

**Antibody Generation**—Chicken mAb HUC750 was generated essentially as described previously (20). To obtain a reaction spectrum for both polymeric and intact OPN, chickens were immunized with polymeric OPN, and the clone was screened with intact OPN. Briefly, after 2-month-old H-B15 inbred chickens were immunized, a phage-displayed library expressing immunoglobulin Fab fragments was constructed from each spleen of the chickens by fusing PCR-amplified immunoglobulin VH and VL regions. After the positive Fab phage clones were concentrated by a few rounds of panning, the Fab clone was finally reconstructed into chicken IgY form.

**Site-directed Mutagenesis**—Site-directed mutagenesis was performed with the QuikChange site-directed mutagenesis kit (Stratagene) as described (21).

**Cell Adhesion Assay**—A cell adhesion assay was performed as described (17) with slight modifications. Briefly, cells were seeded into wells of MaxiSorp enzyme-linked immunosorbent assay plates (Nunc) coated with substrate proteins at 4 °C for overnight and then incubated for 1 h at 37 °C. Attached cells were stained with 0.5% crystal violet and solubilized in 2% Triton X-100 for taking optical density at 595 nm.

**Recombinant Soluble α9β1—FreeStyle 293F cells were co-transfected with cDNAs encoding truncated α9 subunit cloned in pEF6/V5-His and β1 subunit in pcDNA3.1 (Invitrogen) lacking the transmembrane and cytoplasmic domains. After screening with zeocin and neomycin, the V5-His-tagged α9β1 heterodimer was purified from culture supernatant by nickel chelate chromatography. For binding assay, 50 μl of recombinant soluble α9β1 in Tris-buffered saline containing 0.05% Tween 20 and 1 mM MnCl₂ was added at a concentration of 2 μg/ml in each well of an enzyme-linked immunosorbent assay plate coated with various OPN proteins or other substrates (50 μl at indicated concentration) and incubated for 60 min at 37 °C. After washing off the unbound α9β1 with the same buffer, binding was assessed by colorimetric detection with anti-V5 antibody and secondary horseradish peroxidase-labeled antibody.

**Affinity Chromatography**—Affinity chromatography was performed as recommended by the manufacturer. Briefly, Tfn3NRAA was coupled to Sepharose gel (1 ml) in a HiTrap column (GE Healthcare) for 30 min at 25 °C. The affinity matrices were blocked with 0.5 M monooctanalamine, and then biotinylated recombinant soluble α9β1 was applied to the column, which had been equilibrated in column buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM MnCl₂). After the column was washed, bound protein was eluted with 20 mM EDTA. Column fractions were concentrated 20 times with a Nanosep 30K ultrafiltration column (Pall Corp.) and then loaded onto 7.5% SDS-PAGE. 

**REFERENCES**

1. In the present study, we demonstrate that polymeric OPN Attracts Neutrophils Binding to α9β1.
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were coated with 15 \( \mu l \) of substrate at the indicated concentrations. A total of \( 1 \times 10^5 \) of SW480 cells were added to the top of each chamber and then allowed to migrate to the lower chambers containing Dulbecco’s modified Eagle’s medium with 1% fetal bovine serum at 37 °C for 24 h in a humidified atmosphere with 5% \( \text{CO}_2 \). Migrated cells on the bottom side of the membrane were stained with crystal violet for counting.

Horizontal Migration of Neutrophils—Human neutrophils were isolated from peripheral blood by Ficoll-Hypaque density gradient centrifugation followed by 3% dextran sedimentation. Migration was assessed using TAXIScan (GE Healthcare Japan) with an etched silicon substrate and a flat glass plate, which together formed a horizontal, 5-\( \mu m \)-deep microchannel (22). Neutrophils in channels were tracked by time-lapse photography, and the number of cells was counted automatically. When blocking antibody was used, neutrophils were preincubated on ice for 15 min with antibody. A total of \( 5 \times 10^5 \) neutrophils were applied to an edge of the microchannel in one compartment of the chamber with RPMI. To start the assay, 1 \( \mu l \) of polymeric OPN (20 \( \mu g/ml \)), intact OPN (20 \( \mu g/ml \)), or fMLP (\( 10^{-7} \) M) was injected into the opposite compartment with RPMI to form a gradient in the channel.

RESULTS

Recombinant Polymeric and Monomeric Osteopontin Proteins—Anion-exchange chromatography of recombinant polymerized OPN is shown in Fig. 2A. To exclude non-polymerized OPN seen in lanes 1–3, fractions of lanes 4–11 were collected. Purity of the polymeric OPN, intact monomeric OPN, and thrombin-cleaved nOPN were confirmed by Western blotting with mAb HUC750 (Fig. 2B). HUC750 reacted polymeric OPN, intact monomeric OPN, and thrombin-cleaved nOPN and showed that these OPN proteins were uncontaminated. The term “intact OPN” represents intact (full-length) monomeric OPN throughout this report.

Cell Adhesion to Polymeric Osteopontin—To investigate whether integrin \( \alpha 9 \beta 1 \) really acts as a receptor for polymeric OPN, we first examined cell adhesion. \( \alpha 9 \)-transfected SW480 cells, which express \( \alpha 9 \beta 1 \) in addition to two naturally expressed polymeric OPN-binding integrins, \( \alpha 5 \beta 1 \) and \( \alpha v \beta 5 \) (9), were used as described previously (4). Both mock-transfected and \( \alpha 9 \)-transfected SW480 cells adhered well to polymer OPN (Fig. 3A). However, adhesion of mock transfectants was significantly reduced in the presence of anti-\( \alpha 5 \beta 1 \) and anti-\( \alpha v \beta 5 \) antibodies, whereas adhesion of \( \alpha 9 \)-transfected cells was only slightly inhibited. Anti-\( \alpha 9 \beta 1 \) partially inhibited adhesion of \( \alpha 9 \)-transfected cells. When these mAbs against \( \alpha 5 \beta 1 \), \( \alpha v \beta 5 \), and \( \alpha 9 \beta 1 \) were used in combination, adhesion of \( \alpha 9 \) transfection

PAGE in a non-reducing condition. \( \alpha 9 \beta 1 \) on the gel was visualized with horseradish peroxidase-labeled avidin after \( \alpha 9 \beta 1 \) had been transferred onto Immun-Blot a polyvinylidene difluoride membrane (Bio-Rad).

Surface Plasmon Resonance Analysis—Monoclonal antibodies were amine-coupled to the research grade CM5 sensor chip of BIACore 2000 (GE Healthcare). When binding of soluble candidate ligands for \( \alpha 9 \beta 1 \) was assessed, anti-V5 monoclonal antibody was coupled to the sensor chip, and recombinant soluble \( \alpha 9 \beta 1 \) tagged with the V5 sequence was passed over (20 \( \mu l/min \)) to be immobilized on the sensor chip.

Transwell Migration—The undersides of polycarbonate membranes with 8-\( \mu m \) pores of a Transwell insert (Corning)
tants was dramatically reduced. These results indicate that α9β1 mediates cell adhesion to polymeric OPN in addition to α5β1 and αvβ5. Next, we examined adhesion to intact OPN and thrombin-cleaved nOPN, non-ligand and ligand for α9β1, respectively, as control experiments. Mock- and α9-transfected SW480 cells showed modest, comparable levels of adhesion to intact OPN, which was completely abrogated by the combination of anti-α5β1 and anti-αvβ5, whereas anti-α9β1 had no effect (Fig. 3B, left). For thrombin-cleaved nOPN, patterns of adhesion of mock- and α9-transfected SW480 cells in the presence or absence of antibodies was essentially the same as to polymeric OPN (Fig. 3B, right). The loss of adhesion with anti-α5β1 and anti-αvβ5 to intact OPN and the contrasting adhesion to thrombin-cleaved nOPN validate our experimental system and increase confidence that α9β1 mediates cell adhesion to polymeric OPN.

Cell Migration to Polymeric Osteopontin—We next assessed α9β1-mediated cell migration to polymeric OPN in a Transwell migration assay. Fig. 4A shows pictures of the underside of a Transwell membrane. Stained α9-transfected SW480 cells transmigrated onto the underside of membrane coated with intact OPN or polymeric OPN appear as dark dots. The obvious difference illustrates that α9 transfectants migrate in response to polymeric OPN but not to intact OPN. To confirm that this enhanced migration is α9β1-mediated, we analyzed the migration in the presence and absence of anti-OPN receptor integrin antibodies (Fig. 4B). When compared with intact OPN, both mock-transfected and α9-transfected cells migrated onto polymeric OPN, with enhanced migration in α9 transfectants. Migration of the mock transfectants was inhibited by a combination of antibodies against α5β1 and αvβ5, whereas the inhibitory effect of the same combination on α9 transfectants was

FIGURE 5. Binding of recombinant α9β1 to polymeric OPN. A, affinity chromatography. Biotin-labeled recombinant α9β1 was passed over a TNfn3RAA-coupled Sepharose column. Bound protein was eluted with 20 mM EDTA and analyzed by 7.5% polyacrylamide gel under non-reducing conditions. The final fraction washed with column buffer was in lane 1, and elution fractions (one column volume × 3) were in lanes 2–4. The positions of molecular mass markers (in kDa) are shown to the left. B, recombinant α9β1 (2 μg/ml) in Tris-buffered saline containing 1 mM Ca2⁺ and 1 mM Mg2⁺, 1 mM Mn2⁺, or EDTA, as indicated, was allowed to bind to wells of an enzyme-linked immunosorbent assay plate coated with 0.1–30 μg/ml polymeric OPN, TNfn3RAA, or vitronectin. Then, to detect α9β1, anti-V5 mAb was placed in wells followed by secondary antibody and colorimetry analysis. Values represent mean ± S.D. (error bars) of duplicate wells in three independent experiments (n = 6). C, surface plasmon resonance (Biacore) analysis of recombinant α9β1 binding to polymeric OPN. The vertical axis shows the surface plasmon resonance intensity in resonance units (RU). The horizontal axis shows the duration of flow of buffer containing 1 mM Mn2⁺. Recombinant α9β1 (10 μg/ml) was bound to anti-V5 that had been coupled to the sensor chip. Polymeric OPN at a concentration of 3, 10, 30, or 90 μg/ml in buffer was added over the sensor chip at a flow rate of 10 μl/min for 180 s.
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Direct Binding of α9β1 to Polymeric Osteopontin—The α9β1-mediated cell adhesion and migration to polymeric OPN strongly suggest that polymeric OPN is a ligand for α9β1. We next detected direct binding of α9β1 to polymeric OPN. First, binding activity of recombinant α9β1 was analyzed with affinity chromatography over affinity matrices coupled with TNfn3RAA, a well characterized α9β1-specific ligand (17). Two bands in the elution fractions in Fig. 5A (lanes 2–4) correspond to biotin-labeled α9β1 that was eluted with EDTA, indicating that the recombinant heterodimer retains expected cation-dependent ligand binding function. Recombinant α9β1 was incubated in wells of enzyme-linked immunosorbent assay plates coated with polymeric OPN, intact OPN, TNfn3RAA, or vitronectin, an irrelevant integrin ligand. α9β1 bound to polymeric OPN as well as TNfn3RAA in a dose-dependent manner in the presence of Ca$$^{2+}$/Mg$$^{2+}$ or Mn$$^{2+}$ (Fig. 5B, left and middle). No binding to intact OPN or vitronectin in the same condition confirmed the specificity of recombinant α9β1. When the divalent cations were depleted and EDTA was present, bindings of α9β1 to polymeric OPN and TNfn3RAA were completely abolished at all concentrations (Fig. 5B, right). To further explore binding of soluble α9β1 to polymeric OPN, we next performed surface plasmon resonance analysis, with recombinant α9β1 coupled to the sensor chip of the instrument. Response unit curves at 3, 10, 30, and 90 μg/ml polymeric OPN clearly illustrate dose-dependent binding of polymeric OPN to α9β1 (Fig. 5C). The α9β1-OPN complex appeared to dissociate easily as expected for integrin-ligand interactions, as demonstrated by the sharp decline in binding at 180 s, when polymeric OPN is no longer present in the running buffer. These results of binding assays with soluble or immobilized α9β1 indicate that integrin α9β1 specifically binds to polymeric OPN.

Integrin α9β1 Mediates Neutrophil Migration to Polymeric Osteopontin—To explore biological meaning of this receptor-ligand interaction, we next evaluated migration of neutrophils because α9β1 is highly expressed on neutrophils and found to mediate neutrophil migration to VCAM-1 (14). Further, thrombin-cleaved nOPN, a ligand for α9β1, was also reported to attract neutrophils. Images of neutrophils migrating through a horizontal, 5-μm-deep microchannel containing a gradient of a chemoattractant were captured with time-lapse microscopy, and the numbers of neutrophils in the channel were counted automatically. Fig. 6A shows the number of neutrophils that migrated into a channel with gradient of polymeric (supplemental Video 1) or intact OPN over a period of 60 min. A blocking antibody to α9β1 partially, but clearly, inhibited the migration (supplemental Video 2), which was in contrast to the lack of inhibition seen with anti-β2 antibody. Consistently with the previous report (9, 26), only minimal directed migration was seen in the presence of a gradient of intact, monomeric OPN (supplemental Video 3). In a gradient of fMLP (Fig. 6B), neutrophils also migrated (supplemental Video 4), but this effect was not blocked by anti-α9β1 but was partially blocked by anti-β2 (23). In the presence of phosphate-buffered saline in both upper and lower panels, there were slight increases in the number of cells over time, which can be attributed to random migration. Neutrophils migrating through the channels can be seen in the images shown in Fig. 6C. At 0 min, neutrophils were aligned at the upper edge of the channel. After 60 min, cells gradually migrated into higher concentrations of polymeric OPN but not into higher concentrations of intact OPN. Fig. 6D shows the number of cells that migrated per minute into the chamber in a gradient of polymeric OPN, in the presence or absence of antibodies against α9β1 and β2, during the first 10 min of the experiment, when the migration curves were linear (Fig. 6A). The difference in rates of migration under these conditions minimal. However, migration of the α9 transfectants was dramatically inhibited by the addition of anti-α9β1. These results indicate that α9β1 mediates migration onto polymeric OPN.
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FIGURE 7. The SVVYGLR-independent interaction of α9-transfected cells and recombinant α9β1 with polymeric OPN. A, adhesion of α9-transfected SW480 cells to polymeric OPN and thrombin-cleaved nOPN having a wild-type (white columns) or mutated SVVYGLR sequence (gray columns); dYGLR and Y165A represent mutants in which YGLR is deleted and Tyr is replaced with Ala in the SVVYGLR sequence, respectively. Cells were plated in wells coated with 5 μg/ml substrate in the presence of anti-α9β1 and -αβ5. B, binding of recombinant α9β1 to wild-type or mutated polymeric OPN and vitronectin (black column). Recombinant α9β1 was allowed to bind to coated substrates (5 μg/ml) in the presence of 1 mM Mn⁴⁺. Recombinant α9β1 allowed to bind to coated substrates (5 μg/ml) in the presence of 1 mM Mn⁴⁺. Then, to detect α9β1, anti-V5 mAb was placed in wells followed by secondary antibody and colorimetry analysis. Values represent mean ± S.D. (error bars) from three independent experiments with triplicate (n = 9) wells.

FIGURE 8. Impaired recognition of polymeric OPN by SVVYGLR-specific antibody, 34E3. A, specificity of 34E3 was tested by incubating in wells coated with 5 μg/ml thrombin-cleaved nOPN in the presence or absence of 1 mg/ml SVVYGLR synthetic peptide or scrambled peptide, GVRSVLY. Fifty μl of 0.5 μg/ml mAb 34E3 was added to wells and incubated for 60 min followed by colorimetry analysis with horseradish peroxidase-tagged secondary antibody. B, binding of 34E3 or control antibody HUC750 to polymeric OPN, intact OPN, and thrombin-cleaved nOPN. Substrates were coated at 5 μg/ml. C, surface plasmon resonance analysis (Biacore) for binding of 34E3 to polymeric OPN, intact OPN, and thrombin-cleaved nOPN. The three forms of OPNs were passed over the sensor chip to which 34E3 had been coupled at a flow rate of 20 μl/min. The vertical axis indicates surface plasmon resonance intensity (in resonance units (RU)), and the horizontal axis shows the duration of flow of Hanks’ balanced salt solution buffer. In A and B, values represent mean ± S.D. (error bars) from three independent experiments with triplicate (A and B; n = 9) wells. C, a representative reaction curve from three independent experiments.

shows that anti-α9β1, in contrast to anti-β2, significantly inhibited neutrophil migration to polymerized OPN.

Integrin α9β1 Recognizes a Site Other Than SVVYGLR in Polymeric Osteopontin—At the beginning of the present study, we hypothesized that α9β1 should bind to polymeric OPN by recognizing the SVVYGLR site in the same manner to bind to thrombin-cleaved nOPN. The aim of the final part of our investigation was to test this hypothesis. First, we abolished the binding capacity of the SVVYGLR domain by two mutations, which we had previously shown completely abrogate α9β1-mediated adhesion to thrombin-cleaved nOPN (5), dYGLR, and Y165A, in which YGLR was deleted and where Tyr was replaced with Ala, respectively. Although the mutations disrupted the cell adhesive property of thrombin-cleaved nOPN, α9-transfected SW480 cells adhered equally well to wild-type or mutated polymeric OPNs (Fig. 7A). Binding of recombinant α9β1 to polymeric OPN with either mutation was also the same as that seen for wild-type polymeric OPN (Fig. 7B), again indicating that integrin α9β1 recognizes a sequence different from SVVYGLR. To evaluate whether these results were due to exposure of a non-SVVYGLR recognition site(s) on polymeric OPN with greater affinity than the SVVYGLR domain or whether SVVYGLR was not exposed on polymeric OPN, we examined the binding of SVVYGLR-specific antibody 34E3 to polymeric OPN. Binding of 34E3 to thrombin-cleaved nOPN-coated wells was completely abolished in the presence of SVVYGLR peptide but not by the GVRSVLY scrambled peptide (Fig. 8A), confirming that 34E3 recognizes and binds specifically to the SVVYGLR sequence. However, 34E3 did not bind to either polymeric OPN or intact OPN, whereas a control OPN antibody HUC750 bound equally well to all three forms of OPN tested (Fig. 8B). These findings were also confirmed by surface plasmon resonance analysis. Again, polymeric OPN as well as intact OPN did not bind to 34E3 on the sensor chip, whereas thrombin-cleaved nOPN obviously bound 34E3 (Fig. 8C). These results indicate that integrin α9β1 binds to polymeric OPN at a site distinct from SVVYGLR and that the SVVYGLR sequence remains cryptic in polymeric OPN, just as it is in intact monomeric OPN.
DISCUSSION

In the present study, we have found that polymerization of OPN results in a new interaction with the α9β1 integrin, that this interaction induces neutrophil migration, and that α9β1 binds to a site distinct from SVVYGLR, the previously described recognition sequence in thrombin-cleaved OPN. These results demonstrated that OPN changes its property upon polymerization at least in part by means of a new molecular interaction. TG2-catalyzed polymerization of OPN was upon polymerization at least in part by means of a new molecular interaction. TG2-catalyzed polymerization of OPN was first identified in vitro in 1991 (10). Recently in vivo polymerization was demonstrated by Western blotting of a high molecular weight form (12, 13) and by N-terminal sequence (12). Because ligand density and affinity are major determinants of integrin clustering and polymerization increases local density of integrin-binding sequences, it is conceivable that polymerization augments the interaction of cells with OPN. We have in fact previously reported that integrin-mediated cell adhesion, spreading, and migration were enhanced by polymeric OPN associated with intense focal contact formation (9). In the present study, we have identified a new molecular interaction of polymeric OPN and demonstrated at least one physiological consequence: neutrophil chemotaxis.

TG2 is known to modulate cell-matrix interaction by cross-linking several extracellular matrix proteins, including collagen, laminin, and fibronectin, effects generally thought to stabilize tissue integrity (11, 16). In addition, because cross-linking changes physical and chemical properties of a protein, polymerization could cause conformational changes in these or other proteins that result in new molecular interactions (16).

Injection of intact OPN has been demonstrated to induce neutrophil recruitment by independent groups in mice and rats (24, 25). Further, an OPN-neutralizing antibody inhibited neutrophil infiltration into livers in mice with concanavalin A- or lipopolysaccharide-induced hepatitis (26, 27). It is thus clear that OPN contributes to neutrophils in vivo. On the other hand, as we have found, there is little evidence for any chemotactic activity of native monomeric OPN in vitro. In contrast, both thrombin-cleaved nOPN (26) and, in the current study, polymerized OPN, are potent neutrophil chemoattractants. It is thus conceivable that injected or endogenously produced OPN recruits neutrophils as a consequence of one of these post-translational modifications. The only report that claimed to show in vitro migration of neutrophils to native OPN used OPN purified from a macrophage cell line, RAW 264.7 cells (24), a condition that would be susceptible to both thrombin cleavage and TG2-mediated polymerization. It is noteworthy that these two different post-translational modifications render OPN accessible to binding to the same receptor, α9β1.

The totally unaffected adhesion and binding of α9-transferrants and recombinant α9β1 to polymeric OPN with disrupted SVVYGLR sequence demonstrated that SVVYGLR in polymeric OPN is not a recognition site of α9β1. Furthermore, the absence of binding of SVVYGLR-specific antibody to polymeric OPN suggests that the SVVYGLR sequence is not even exposed on the surface of the polymer. We are not certain why two different post-translational modifications of OPN, cleavage and polymerization, result in the employment of the same receptor through distinct binding sites. Nonetheless, integrin α9β1 appears to be critical for chemotactic responses of neutrophils to OPN.

One important role for the interaction of α9β1 with polymerized OPN might be to concentrate neutrophils at sites of OPN polymerization, ensuring that their immune products and activities remain at this site, whereas minimizing unnecessary injury to the other sites. Because both OPN and TG2 are upregulated at sites of injury and inflammation, it is conceivable that polymeric OPN could be generated in response to injury. Thrombin-cleaved nOPN could also be generated at sites of inflammation, so it will be important to investigate the kinetics and location of these two post-translational modifications under various conditions in vivo and the relative contribution of each to neutrophil recruitment and/or retention.

In conclusion, we have identified a de novo molecular interaction upon polymerization of OPN and concomitant induction of neutrophil migration. Our findings illustrate that OPN gains a new function through polymerization and open new avenues to understand the biological significance of OPN polymerization and its potential role in regulating tissue inflammation.

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