Expression of wild-type and mutated rabbit osteopontin in *Escherichia coli*, and their effects on adhesion and migration of P388D1 cells

Kaei NASU, Tetsuya ISHIDA, Mihoko SETOGUCHI, Yasunori HIGUCHI, Shin'ichiro AKIZUKI and Shunsuke YAMAMOTO*
Department of Pathology, Oita Medical University, Hasama-machi, Oita 879-55, Japan

Recombinant wild-type rabbit osteopontin (rOP) and the protein with an aspartate-to-glutamate transposition induced by a point mutation in the rabbit OP cDNA within the Gly-Arg-Gly-Asp-Ser (GRGDS) sequence were expressed in *Escherichia coli* and purified to homogeneity. P388D1 cells bound rOP in a saturable manner. rOP induced adhesion and haptotaxis of P388D1 cells, whereas mutated rabbit OP (rOPmut) did not. Anti-rOP IgG $F(ab')_2$ and synthetic GRGDS peptide inhibited rOP-mediated adhesion and haptotaxis of P388D1 cells. Fibronectin (FN)-mediated adhesion of P388D1 cells was markedly inhibited in the presence of fluid-phase rOP. Adhesion of P388D1 cells to rOP was significantly inhibited by anti-[α-subunits of VLA4 (α_4) and VLA5 (α_5)] monoclonal antibodies (mAbs), but not by anti-[α-subunit of vitronectin (VN) receptor (α_V) or Mac-1 (α_M)] mAb. Adhesion of P388D1 cells to FN and VN was significantly inhibited by anti-α_V mAb but not anti-α_4, -α_5 or -α_M mAb. Haptotaxis of P388D1 cells to rOP was significantly inhibited by anti-α_V mAb, but not by anti-α_4, $-\alpha_5$ and α_M mAbs, whereas that to FN showed no inhibition with all three mAbs. Haptotaxis of P388D1 cells to VN was significantly inhibited by anti-α_5 and -α_V mAbs but not by anti-α_4 and -α_M mAbs. Similar features of inhibition of adhesion and haptotaxis of P388D1 cells to human OP were observed by mAbs. rOP had no chemotactic effect on P388D1 cells. Significant polymorphonuclear leucocyte migration was observed 3–12 h after intradermal injection of rOP into rabbits.

INTRODUCTION

Cell–matrix interactions are essential for cell adhesion and migration, wound healing, embryogenesis, cell differentiation and polarity [1]. These interactions are mediated by adhesion of cell-surface receptors, particularly integrins, to adhesive proteins containing Arg-Gly-Asp (RGD) tripeptide sequences, although non-RGD sites also appear to play a role in cell adhesion. A variety of RGD-containing proteins such as fibronectin (FN) [2,3], vitronectin (VN) [4,5], von Willebrand factor [6], collagens [7] and thrombospondin [8] have been reported. These molecules are generally large and contain many functional sites other than those for cell adhesion.

Osteopontin (OP) is an RGD-containing protein expressed in a variety of cell types including bone, macrophages, activated T-cells, placenta, kidney, nerve and tumour cells. Although the function of OP has not been fully elucidated, several previous experiments have indicated a role in resistance to Rickettsia tsutsugamushi, tumorigenicity and adherence in bone and kidney cells [9–14]. These reports suggest that OP has diverse functions in different cell types. Furthermore, it is the smallest RGD-containing protein yet identified, and its matrix adhesiveness may be much more limited than that of FN which is approximately six times larger. The structure of OP around RGD amino acids is closely related to that of FN, and OP has been reported to bind to a receptor integrin $\alpha_V\beta_3$, that is also bound by FN, although the latter also binds to other integrins [15,16]. Therefore it is possible that OP modules the function of FN.

We have previously described the cloning and analysis of OP complementary and genomic DNAs [17,18]. In the present paper, we describe the role of OP in adhesion and haptotactic migration of a murine macrophage cell line, P388D1, using high-quality recombinant wild-type and mutated OP.

EXPERIMENTAL

Materials

Restriction enzymes were purchased from Takara (Kyoto, Japan), Toyobo (Osaka, Japan) and Wako Pure Chemicals (Osaka, Japan). DNA ligation kit and reverse transcriptase were obtained from Takara, and bovine alkaline phosphatase was obtained from Boehringer-Mannheim (Mannheim, Germany). Natural human OP (hOP) prepared from human milk was purchased from Cosmo-Bio (Tokyo, Japan). Anti-[α-subunit of Mac-1 (α_M)] monoclonal antibody (mAb) [19] was obtained from Serotec (Oxford, U.K.), anti-[α-subunit of VLA4 (α_4)] mAb [20] was from Seikagaku Kogyo (Tokyo, Japan) and anti-[α-subunit of VLA5 (α_5)] mAb [20] and anti-[α-subunit of VN receptor (α_V)] mAb [21] were from Sumitomo Denko (Osaka, Japan). The radioactive nucleotide [α-^{32}P]dCTP (3000 Ci/mM) and $Na^{125}I$ (100 mCi/ml) were obtained from DuPont–New England Nuclear (Boston, MA, U.S.A.). Chemicals used for DNA sequencing were obtained from Toyobo. X-ray film (XAR-351) was obtained from Kodak (New Haven, CT, U.S.A.).

Preparation of mRNA and cloning of rabbit OP cDNA

Peritoneal macrophages were obtained from rabbits 4 days after intraperitoneal injection of 1 % casein. The cells were suspended in Dulbecco's modified Eagle's medium (DMEM) and incubated in polystyrene Petri dishes at 37 °C for 1 h. After removal of non-adherent cells by decantation, the adherent cells were disrupted

Abbreviations used: OP, osteopontin; rOP, recombinant osteopontin; hOP, human osteopontin, rOPmut, mutated rabbit osteopontin; FN, fibronectin; VN, vitronectin; mAb, monoclonal antibody; DMEM, Dulbecco's modified Eagle's medium; 1 × SSC, 0.15 M NaCl plus 0.015 M sodium citrate, $2H_2O$ pH 7.0; IPTG, isopropyl β-D(−)-thiogalactopyranoside.

* To whom correspondence should be addressed.

The sequence data will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under accession number D16544.

```
OC-1  SIESHDHMDDIDEDEDDDHVDNR 100
OP    *******V**************Q 100

OC-1  DSNESDDADHPDDSHHSDESHQS 123
OP    ************************ 123

OC-1  DESDE---VTVYPTEDAATTVFT 143
OP    *****SDE*************** 146

OC-1  EVVPTVETYDGRGDSVAYRLKRS 166
OP    *******I**************** 169
```

Figure 1 Differences in amino acid sequences between the previously published rabbit OP (OC-1) and that deduced from the present rabbit OP cDNA

Amino acid positions are indicated on the right. Asterisks indicate identity.

with 6 M guanidinium isothiocyanate, total RNA was isolated by CsCl centrifugation, and polyadenylated RNA was selected by oligotex-dT30 (Daiichi Pure Chemicals, Tokyo, Japan).

cDNA was synthesized from polyadenylated RNA using reverse transcriptase followed by *Escherichia coli* DNA polymerase I as previously described [22], and then ligated into λgt10. The λgt10 rabbit library was then screened using a nick-translated ^{32}P-labelled cDNA for mouse OP as a probe. After hybridization, membranes were washed for 20 min at room temperature with 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, $2H_2O$, pH 7.0) containing 0.1% SDS and then at 55 °C with 1 × SSC containing 0.1% SDS for 90 min. Hybridization-positive clones were purified and amplified to isolate recombinant phage DNA. Two cDNA clones were isolated and inserted into the *Eco*RI site of pUC118. One of the pUC118 cDNA clones, designated 10-2, spans 1080 bp containing the entire open reading frame encoding 314 amino acids flanked by 86 bp of the 5′ and 52 bp of the 3′ non-coding sequences. The nucleotide sequence has been deposited in the GenBank/EMBL/DDBJ Nucleotide Sequence Databases under accession number D16544. The predicted protein sequence matched the rabbit OP cDNA isolated from an osteoclast cDNA library [23] except for six amino acid residues (Figure 1).

Construction of recombinant rabbit OP (rOP)-expressing plasmids

The pUC118 cDNA clone 10-2 linearized with *Bam*HI and *Pst*I and the 5′ end of the cDNA fragment were deleted with exonuclease III followed by mung bean nuclease. The 5′-deleted DNA (10-2d) devoid of the region encoding the putative signal peptide was made blunt-ended by incubation with the Klenow fragment of *E. coli* DNA polymerase I, ligated and propagated. The 10-2d insert was cleaved with *Eco*RI and *Hin*dIII, followed by Klenow treatment, and ligated into pET-11a vector [24] which had been cleaved with *Bam*HI and blunt-ended. *E. coli* K12 strain HB101 was used as the host for initial cloning and maintenance of the resulting plasmids.

To construct a mutated clone, 10-2d was subcloned into the *Eco*RI–*Hin*dIII site of the M13mp18 polylinker, and single-stranded DNA was obtained. The synthetic oligonucleotide used for mutagenesis was 5′-GACGGCCGTGGTGAAAGTG-TG-3′, which changes the wild-type amino acid sequence GRGDS into GRGES. After annealing of the oligonucleotide to the single-stranded DNA, extension of the annealed oligonucleotide to generate double-stranded DNA was performed using the thionucleotide dCTPαS. The resulting phosphothioate DNA was digested with *Nci*I and subjected to exonuclease III treatment to remove the non-mutant sequence, followed by repolymerization using DNA polymerase I to generate homoduplex Mutant DNA, designated rOPmut.

Bacterial growth and induction protocol

Rabbit OP-expressing plasmids were propagated in *E. coli* strain BL21(DE3), a derivative of BL21 cells (F^- ompT r_B^- m_B^-) [24]. Cultures were grown at 37 °C for 4 h in M9ZY medium (100 ml) supplemented with ampicillin (50 μg/ml), and rabbit OP expression was induced by addition of 1 mM isopropyl β-D(−)-thiogalactopyranoside (IPTG) when the medium containing the cells reached an absorbance at 600 nm of about 0.5–0.6. Induction was terminated 1–3 h later by rapidly cooling the cells to 0 °C on crushed ice.

Analysis of bacterial extracts and purification of rOP

Bacterial cells were harvested by centrifugation, suspended in PBS containing 0.05% Tween 20 and disrupted by sonication. Sonicates were spun at 17000 *g* for 5 min and the supernatants and pellets were recovered for analysis. Aliquots were lysed in SDS loading buffer (10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 50 mM Tris/HCl, 0.25% Bromophenol Blue, pH 6.8) and heated for 10 min at 100 °C. Proteins separated on 12% polyacrylamide gels were either stained with Coomassie Brilliant Blue or were electrophoretically transferred to nitrocellulose membranes and allowed to react with goat anti-rOP. The immunoblots were washed, and allowed to react with horseradish peroxidase-conjugated rabbit anti-goat IgG (heavy- and light-chain-specific; Cappel, West Chester, PA, U.S.A.) followed by substrate.

For preparative scale, cultures were grown in 1 litre of broth. Bacterial cells were harvested by centrifugation and suspended in PBS containing 0.05% Tween 20, lysed by sonication and centrifuged. The supernatant was treated with streptomycin (1 mg/ml) to precipitate DNA, and precipitated in the presence of 30–50%-satd. $(NH_4)_2SO_4$ at 4 °C. After centrifugation, the precipitates were dissolved in PBS and subjected to HPLC (Synchropak AX1000; SynChrom, Lafayette, IN, U.S.A.) equilibrated in 16 mM sodium phosphate, pH 7.6. Some 40 mg of purified rOP was isolated from 6 ml of cell mass from a 1-litre bacterial culture.

Preparation of anti-rOP antibody

Anti-rOP sera were obtained from a goat which received multiple subcutaneous injections of rOP emulsified with Freund's complete adjuvant in the footpads, both thighs and the back and nape of the neck. After 1 month, the goat was partially bled and blood samples were subjected to ELISA for determination of antibody titre. These antisera were applied to a Protein A–cellulofine column (Seikagaku Kogyo). The antibody fraction was eluted with 0.05 M sodium acetate buffer, pH 4.0, containing 0.15 M NaCl. $F(ab')_2$ of goat IgG was prepared as described previously [25]. An anti-rOP affinity column was prepared by conjugating anti-rOP IgG to CNBr-activated cellulofine.

Estimation of binding assay

rOP, rOPmut, FN and VN were iodinated with carrier-free ^{125}I by the Iodogen method (Pierce, Rockford, IL, U.S.A.) [26]. ^{125}I-labelled proteins were separated on Sephadex G-25 columns (Pharmacia LKB, Uppsala, Sweden), and specific binding was investigated using P388D1 cells. P388D1 cells cultured in DMEM supplemented with 0.1% BSA (Sigma, St. Louis, MO, U.S.A.)

were used at a density of $5 \times 10^4/250$ μl per well in 48-well plates for experiments. The cells were incubated at 20 °C for 90 min with medium containing the indicated amounts of ^{125}I-labelled protein and 0.2% BSA, washed four times with PBS, and solubilized with 250 μl of 1% SDS [27]. Total binding was determined by measuring the radioactivity of the resulting lysates. Non-specific binding was determined by the addition of a 400-fold excess of unlabelled protein.

Estimation of cell adhesion

Tests for cell adhesion were performed as previously described [28]. Culture plates containing 96 wells were precoated overnight at 4 °C with 0.2–10 μg/ml FN, VN, rOP, rOPmut or hOP in PBS, pH 7.3, and non-specific sites were blocked with BSA (1 mg/well) in PBS for 2 h at 4 °C. P388D1 cells (1×10^6/ml) were suspended in DMEM, and 100 μl of the cell suspension was introduced into each well. Plates were incubated under 5% CO_2 for 90 min at 37 °C. After one wash with PBS, the adhering cells were fixed with 3% paraformaldehyde. The cells were then rinsed with 0.1 M borate buffer, pH 8.5, and then stained with 1% Methylene Blue. After four washes with 0.1 M borate buffer, the stained cells were lysed with 1 M HCl, and subjected to photocytometric analysis at A_{595}. For the inhibition assay, 96-well culture plates pretreated as above with FN (5 μg/ml), VN (5 μg/ml), rOP (5 μg/ml) or hOP (5 μg/ml), followed by rinsing with PBS were subjected to adhesion assay in the presence of various amounts of FN, VN, rOP, rOPmut, synthetic GRGDS peptide, anti-rOP IgG F(ab')$_2$ or anti-integrin mAbs. All experiments were performed in triplicate, and the data were averaged.

Estimation of haptotaxis

Haptotaxis, the ability of cells to detect and move up gradients of substratum adhesiveness, was assayed in a modified Boyden chamber [29] by the method of McCarty et al. [30]. The lower surfaces of 5 μm-pore-size polycarbonate filters (Nuclepore, Pleasanton, CA, U.S.A.) were coated with 0.01–10 μg/filter FN, VN, rOP, rOPmut or hOP, and dried overnight at room temperature. The filters were vigorously washed with PBS and again dried. The treated filter was placed between the upper and lower compartments of the chamber in which the lower compartment had previously been filled with DMEM containing 0.1% BSA. After the filter had been set and 1 ml of P388D1 cells (2×10^5/ml) in DMEM containing 0.1% BSA had been added, the chamber was placed in a CO_2 incubator for 4 h at 37 °C. For the inhibition assay, the filters pretreated as above with FN (2 μg/filter), VN (2 μg/filter), rOP (2 μg/filter) or hOP (5 μg/filter) were subjected to a migration assay in the presence of various amounts of FN, VN, rOP, rOPmut, synthetic GRGDS peptide, anti-rOP IgG F(ab')$_2$ or anti-integrin mAbs. All experiments were performed in triplicate, and the data were averaged.

Estimation of leucocyte migration *in vivo*

rOP, rOPmut, PBS or a preparation after elution of rOP from an anti-rOP affinity column (300 ng each) was intradermally injected into rabbits, and dermal tissue was removed at intervals of 3, 6, 12 and 24 h. After fixation in 10% formalin, tissue was embedded in paraffin, sectioned, and stained with haematoxylin and eosin.

RESULTS

Expression and purification of rOP and rOPmut

Expression of rOP was achieved by using the expression vector

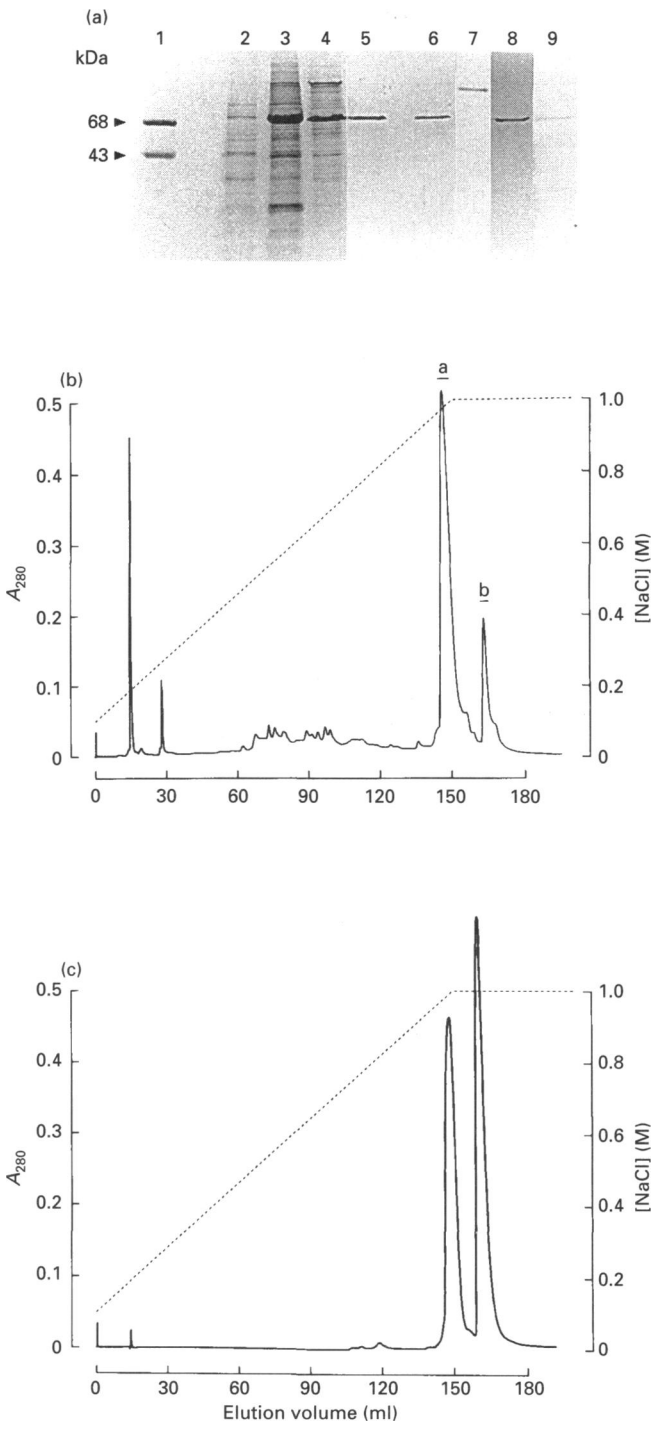

Figure 2 SDS/PAGE analysis of proteins from *E. coli* transformed with pETrOPx and elution profiles of rOP by anion-exchange HPLC

(**a**) SDS/PAGE analysis of proteins from *E. coli* transformed with pETrOPx. Lanes 1–8, SDS/PAGE; lane 9, Western blot using anti-rOP antibody. The lanes contained the following: 1, molecular-mass markers (the upper and lower bands represent BSA and ovalbumin markers respectively); 2, total cell extract before induction; 3, total cell extract after induction; 4, sample after $(NH_4)_2SO_4$ precipitation; 5, major peak of rOP after HPLC; 6, rOPmut after HPLC; 7, minor peak of rOP after HPLC (non-reducing conditions); 8, minor peak of rOP after HPLC (reducing conditions); 9, rOP detected by Western blotting. (**b**) Elution profiles of rOP by anion-exchange HPLC. A sample obtained by 30–50% $(NH_4)_2SO_4$ saturation was loaded on to a Synchropak AX1000 column in 20 mM Tris/HCl, pH 7.6. Fractions (0.5 ml) were collected at a flow rate of 3 ml/min. Bars show fractions pooled for SDS/PAGE and amino acid analysis. (**c**) The major peak eluted during the initial HPLC was rechromatographed and found to yield a minor peak very similar to that found during the initial HPLC.

Table 1 Analysis of amino acid composition of HPLC fractions of rOP

The experimental results are means of five different preparations.

Amino acid	Theoretical	Experimental	
		Fraction a	Fraction b
Asp + Asn	53	53.0	53.0
Thr	13	12.2	12.1
Ser	48	48.0	48.0
Glu + Gln	55	55.0	55.0
Pro	15	10.9	9.2
Gly	8	8.3	8.6
Ala	18	18.0	18.0
Cys	1	0.3	0.3
Val	20	20.0	21.0
Met	6	4.7	4.7
Ile	7	6.5	6.7
Leu	15	15.0	15.5
Tyr	6	5.6	5.7
Phe	3	2.9	3.1
His	21	20.8	21.0
Lys	15	15.9	14.9
Trp*	2		
Arg	11	10.2	10.4

* Tryptophan was not determined as it is destroyed by the hydrolytic conditions used.

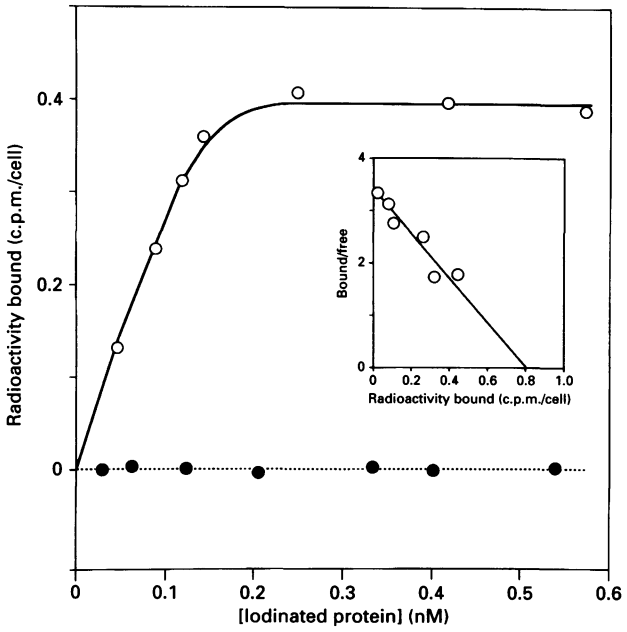

Figure 3 Specific binding of ^{125}I-labelled rOP and ^{125}I-labelled rOPmut to P388D1 cells

The cells were incubated at 20 °C for 90 min in medium containing the indicated amounts of ^{125}I-labelled protein. Non-specific binding was determined by the addition of a 400-fold excess of unlabelled protein and was subtracted from total binding. Each value represents the mean of triplicate wells. ○, rOP; ●, rOPmut.

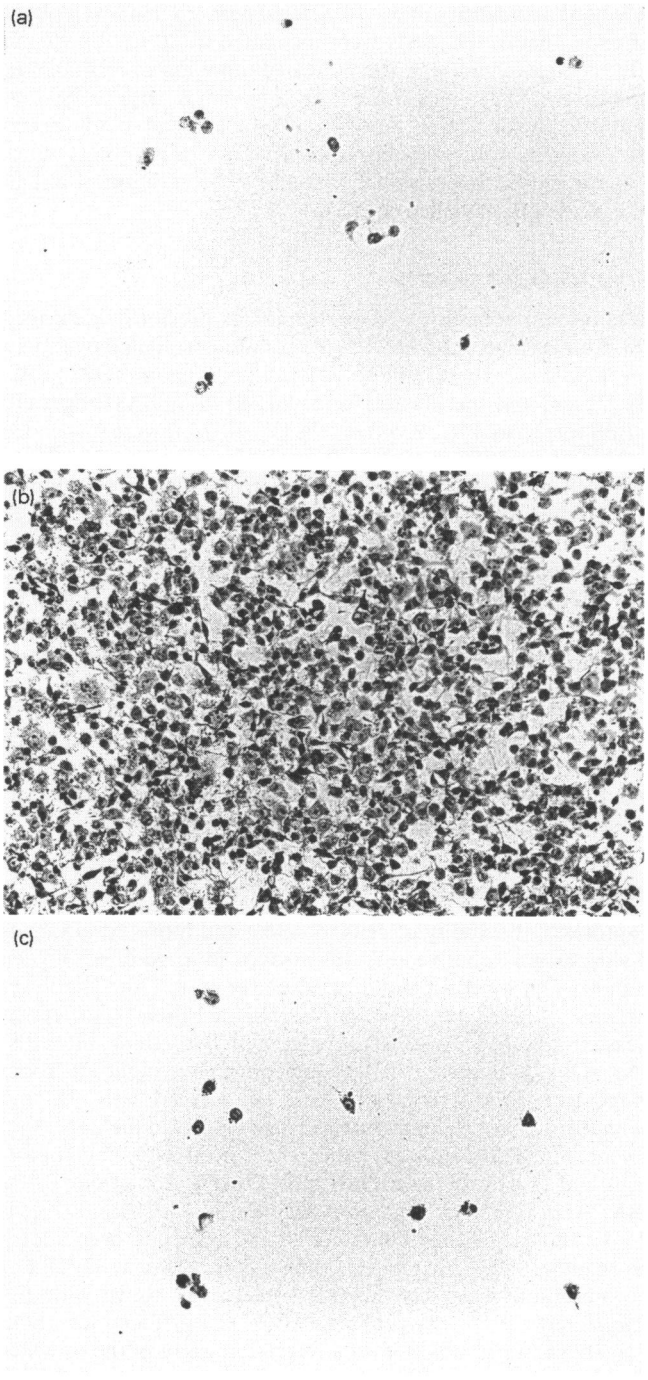

Figure 4 Photomicrographs of cell adhesion to rOP- and rOPmut-coated plastic Petri dishes

P388D1 cells in DMEM were cultured at 37 °C for 90 min. (**a**) No coating; (**b**) rOP coating; (**c**) rOPmut coating. Magnification × 50.

pET-11a containing the T7 lac promoter. BL21(DE3) cells transformed with pET vector containing the rabbit OP sequence were incubated in the presence of IPTG for 1–3 h at 37 °C. The induced cells were pelleted, lysed with 1 × SDS loading buffer and subjected to SDS/PAGE. A protein with an apparent molecular mass of 70 kDa was specifically induced (Figure 2a). The predicted molecular mass of rabbit OP is 35455 Da, and no glycosylation has been found on rOP (results not shown). In addition the amino acid composition of purified rOP was very similar to that of rabbit OP deduced from its cDNA sequence

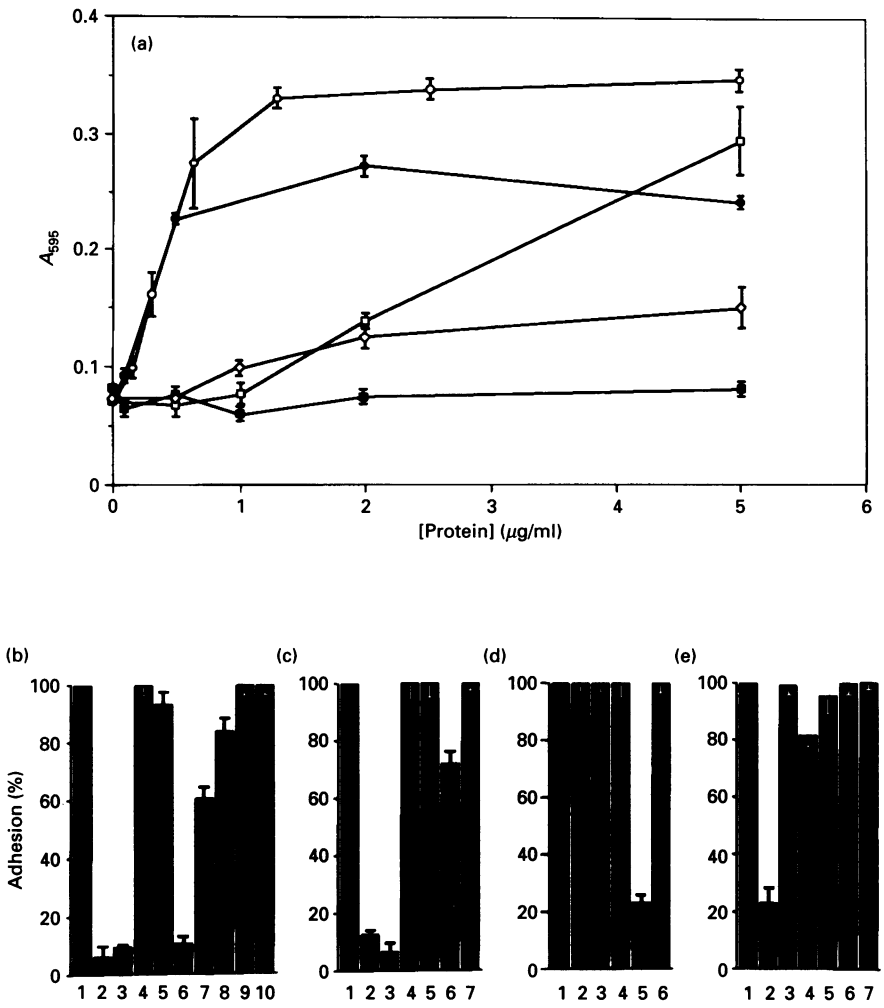

Figure 5 Adhesion of P388D1 cells to FN-, VN-, rOP-, rOPmut- and hOP-coated plastic micro wells, and effects of various proteins and antibodies on binding of P388D1 cells to rOP- FN-, VN- and hOP-coated wells

(**a**) Adhesion of P388D1 cells to FN- (□), VN- (●), rOP- (○), rOPmut- (■) and hOP (◇)-coated plastic micro wells. P388D1 cells in DMEM were cultured in FN-, VN- rOP-, rOPmut- and hOP-coated 96-well plates at 37 °C for 90 min. After being washed, cells were fixed with paraformaldehyde, stained with 1% Methylene Blue, lysed with 1 M HCl, and subjected to photocytometric analysis at an absorbance of 595 nm. (**b**), (**c**), (**d**) and (**e**), Effects of rOP, rOPmut, GRGDS peptide, FN, VN, anti-rOP IgG $F(ab')_2$ and anti-integrin antibodies on adhesion of P388D1 cells to rOP-, FN-, VN- and hOP- (5 μg/ml) coated wells. (**b**) Effects of various proteins and antibodies on binding of P388D1 cells to rOP-coated wells: 1, control; 2, fluid-phase GRGDS peptide (5 μg/ml); 3, fluid-phase FN (5 μg/ml); 4, fluid-phase VN (5 μg/ml); 5, fluid-phase rOPmut (5 μg/ml); 6, anti-rOP IgG $F(ab')_2$; 7, anti-α_4; 8, anti-α_5; 9, anti-α_V; 10, anti-α_M. (**c**) Effects of various proteins and antibodies on binding of P388D1 cells to FN-coated wells: 1, control; 2, fluid-phase GRGDS peptide (5 μg/ml); 3, fluid-phase rOP (5 μg/ml); 4, anti-α_4; 5, anti-α_5; 6, anti-α_V; 7, anti-α_M. (**d**) Effects of various proteins and antibodies on binding of P388D1 cells to VN-coated wells: 1, control; 2, fluid-phase rOP (5 μg/ml); 3, anti-α_4; 4, anti-α_5; 5, anti-α_V; 6, anti-α_M. (**e**) Effects of various proteins and antibodies on binding of P388D1 cells to hOP-coated wells: 1, control; 2, fluid-phase FN (5 μg/ml); 3, fluid-phase VN (5 μg/ml); 4, anti-α_4; 5, anti-α_5; 6, anti-α_V; 7, anti-α_M.

(Table 1). rOP may exist as a dimer, although it is curious that it is not resolved into the monomeric form on reducing SDS/polyacrylamide gels.

Maximal rOP expression was obtained 2.5 h after IPTG induction, and analysis of samples obtained from a larger-scale preparation showed that rOP was present in a soluble form. The cleared supernatant obtained by centrifugation of sonicated bacterial extracts was subjected to streptomycin and $(NH_4)_2SO_4$ precipitation, followed by HPLC fractionation. Two main peaks were found. The major eluate was rechromatographed and yielded a minor peak very similar to that observed during the initial HPLC (Figures 2b and 2c). Furthermore, we have found that the content of the minor component varies with time of dialysis against PBS.

The amino acid composition of the major and minor rechromatographed eluates is shown in Table 1 (fraction a and b respectively). The amino acid composition of the major eluate was almost identical with both that of rOP predicted from the sequence of the rabbit OP cDNA and that of the minor eluate. SDS/PAGE analysis showed that the mobility of the minor eluate was higher under reducing conditions than non-reducing conditions, and was similar to that of the major eluate (Figure 2a), suggesting that the minor component comprised aggregates of rOP.

Next, rOPmut was produced by a single nucleotide substitution of an A for a C residue in the region encoding GRGDS, which changed the wild-type amino acid sequence GRGDS into GRGES. SDS/PAGE analysis showed rOPmut to have an electrophoretic mobility identical with that of wild-type rOP (Figure 2a).

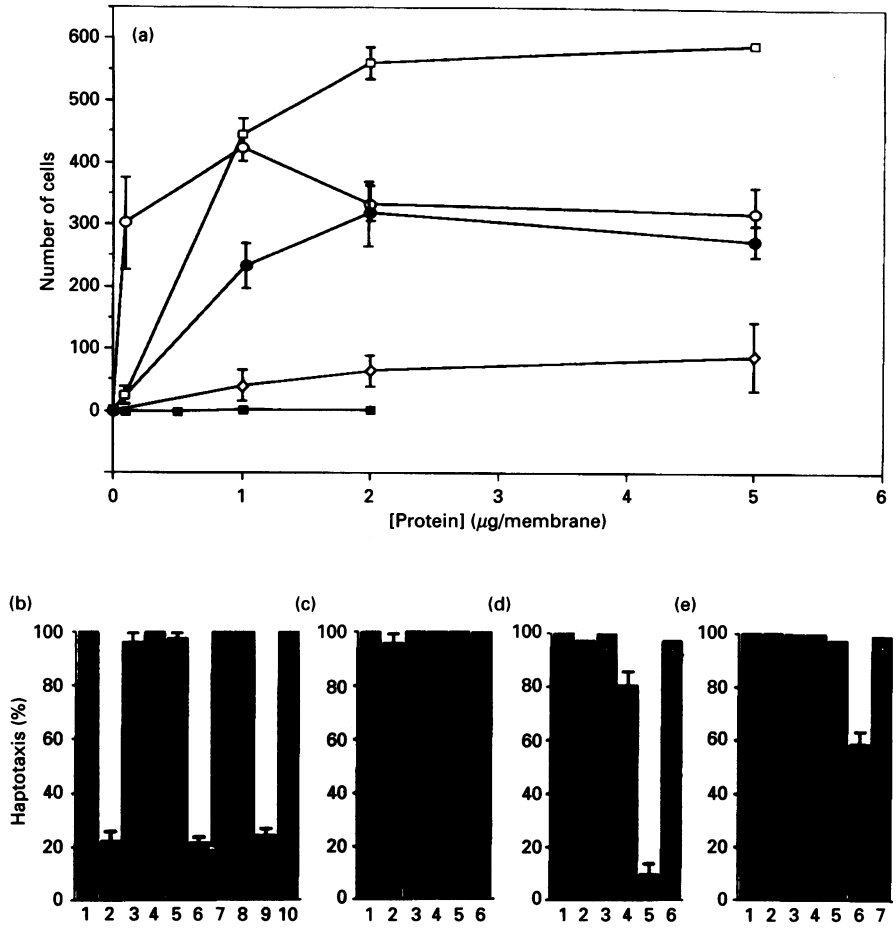

Figure 6 Haptotaxis of P388D1 cells to FN-, VN-, rOP-, rOPmut- and hOP-coated filters, and effects of various proteins and antibodies on haptotactic migration of P388D1 cells to rOP-, FN-, VN- and OP-coated filters

(**a**) Haptotaxis of P388D1 cells to FN- (□), VN- (●), rOP- (○), rOPmut- (■) and hOP (◇)-coated filters. Polycarbonate filters were precoated to insolubilize FN, VN, rOP, rOPmut and hOP on the lower surface. After extensive washing, the filters were used in Boyden chamber migration assays. Data were obtained by counting the cells in five random medium-power fields (× 200) on the lower surface of filters from triplicate microchambers. (**b**), (**c**), (**d**) and (**e**) Effects of rOP, rOPmut, GRGDS peptide, FN, VN, anti-rOP IgG ($F(ab')_2$ and anti-integrin antibodies on haptotactic migration of P388D1 cells to rOP-, FN-, VN- and hOP- (2 μg/filter) coated filters. (**b**) Effects of various proteins and antibodies on haptotactic migration of P388D1 cells to rOP-coated filters: 1, control; 2, fluid-phase GRGDS peptide (2 μg/ml); 3, fluid-phase FN (2 μg/ml); 4, fluid-phase VN (2 μg/ml); 5, fluid-phase rOPmut (2 μg/ml); 6, anti-rOP IgG $F(ab')_2$; 7, anti-α_4; 8, anti-α_5; 9, anti-α_V; 10, anti-α_M. (**c**) Effects of various proteins and antibodies on haptotactic migration of P388D1 cells to FN-coated filters: 1, control; 2, fluid-phase rOP (2 μg/ml); 3, anti-α_4; 4, anti-α_5; 5, anti-α_V; 6, anti-α_M. (**d**) Effects of various proteins and antibodies on haptotactic migration of P388D1 cells to VN-coated filters: 1, control; 2, fluid-phase rOP (2 μg/ml); 3, anti-α_4; 4, anti-α_5; 5, anti-α_V; 6, anti-α_M. (**e**) Effects of various proteins and antibodies on haptotactic migration of P388D1 cells to hOP-coated filters: 1, control; 2, fluid-phase FN (2 μg/ml); 3, fluid-phase VN (2 μg/ml); 4, anti-α_4; 5, anti-α_5; 6, anti-α_V; 7, anti-α_M.

Binding assay of rOP and rOPmut

P388D1 cells were tested for FN-, VN-, rOP- and rOPmut-specific binding. With increased concentrations of ^{125}I-labelled rOP, specific binding increased in a saturable fashion. Scatchard analysis of the binding data of ^{125}I-labelled rOP to P388D1 cells showed a single linear regression line (Figure 3), indicating a single set of binding sites on P388D1 cells (4.1×10^3 sites/cell) and a binding affinity of 2.35×10^{-10} M. rOPmut-specific binding was not observed (Figure 3). Both FN- and VN-specific binding increased in a saturable fashion with increased concentrations of ^{125}I-labelled proteins (results not shown).

Effect of rOP on P388D1 cell adhesion

P388D1 cells adhered weakly to non-treated and rOPmut (5 μg/ml)-coated 96-well culture plates; approx. 99% of cells did not adhere to the plates by 90 min after seeding, and the remaining 1% began to adhere slightly (Figures 4a and 4c). In contrast, most of the cells were spread over the plates precoated with rOP (5 μg/ml) by 90 min after seeding (Figure 4b).

We quantified adhesion by photometrically monitoring lysates of adhering cells stained with Methylene Blue. As shown in Figure 5(a), dose-dependent adhesion of P388D1 cells was observed with FN, VN and rOP. rOPmut containing the GRGES sequence showed no adhesive activity. Adhesion of P388D1 cells by rOP was inhibited by goat anti-rOP IgG $F(ab')_2$ (Figure 5b). To determine whether the GRGDS site was involved in the adhesion, we performed inhibition tests using synthetic GRGDS peptide (5 μg/ml) and rOPmut (5 μg/ml). Adhesion of P388D1 cells by FN, VN and rOP was completely inhibited in the presence of GRGDS, but was not inhibited in the presence of rOPmut (Figures 5b, 5c and 5d).

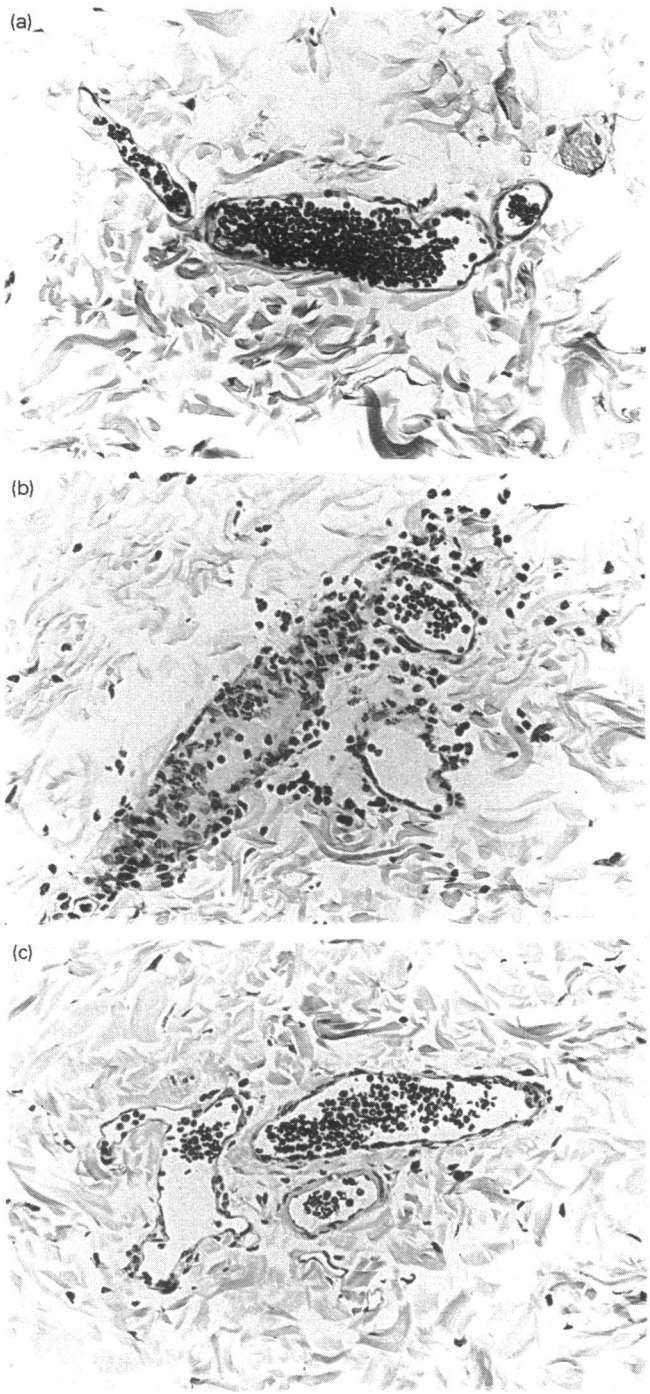

Figure 7 Effects of rOP on leucocyte migration *in vivo*

(**a**) 3 h after intradermal injection of PBS; (**b**) 3 h after intradermal injection of 300 ng of rOP; (**c**) 3 h after intradermal injection of 300 ng of rOPmut. Magnification ×100.

To investigate the possibility that GRGDS-mediated adhesion by FN, VN and rOP may be mutually inhibitive, we analysed the adhesion to FN and VN in the presence of rOP, and vice versa. The results clearly show that rOP inhibited adhesion of P388D1 cells to FN, and that FN inhibited adhesion to rOP (Figures 5b and 5c), whereas rOP did not inhibit adhesion to VN, and VN did not inhibit adhesion to rOP (Figures 5b and 5d).

Osteoclasts have been shown to bind to bone matrix via integrin $\alpha_v\beta_3$ and bone sialoprotein for attachment [31]. To determine what types of integrin receptors are involved in adhesion of P388D1 cells, adhesion assays were performed in the presence of anti-integrin receptor mAbs. Adhesion of P388D1 cells to rOP was significantly inhibited by anti-α_4 or -α_5 mAb, but not by anti-α_v or -α_M mAb (Figure 5b). Adhesion of P388D1 cells to FN and VN was significantly inhibited by anti-α_v mAb but not by anti-α_4, -α_5 or -α_M mAb (Figures 5c and 5d).

Natural OP is extensively glycosylated. We next examined the effect of hOP, prepared from milk, on adhesion of P388D1 cells. Dose-dependent adhesion of P388D1 cells was observed with hOP, although the level of adhesion is lower than that to rOP (Figure 5a). This may be due to glycosylation or species differences. Next, we examined features of inhibition by FN, VN and mAbs. FN inhibited adhesion to hOP, whereas VN did not. Adhesion of P388D1 cells to hOP was significantly inhibited by anti-α_4 but not by anti-α_v or -α_M. The effect of anti-α_5 was not significant (Figure 5e). These effects of FN, VN and mAbs on adhesion of P388D1 cells to hOP were similar to those to rOP.

Effects of rOP on haptotactic migration of P388D1 cells

P388D1 cells show haptotactic migration by sensing a gradient of adhesion molecules on the matrix surface [30]. Nuclepore filters were precoated with FN, VN, rOP or rOPmut (0.01–5 μg/filter) to bind the protein to the lower surface, and dried overnight at room temperature. After extensive washing of the filters, haptotactic assays were performed using modified Boyden chambers. As shown in Figure 6(a), P388D1 cells showed dose-dependent migration on the FN-coated filters. Significant migration was also observed, albeit to a lesser extent on the VN- and rOP-coated filters. Such migration of P388D1 cells was markedly reduced when filters precoated with rOP on both sides were used, although some random migration was observed. No haptotactic migration was observed with rOPmut-coated filters (Figure 6a). Haptotactic migration of P388D1 cells by rOP was inhibited by goat anti-rOP IgG F(ab')$_2$ (Figure 6b). To investigate whether the GRGDS site is involved in haptotactic migration, we performed inhibition assays using synthetic GRGDS peptide, and found that rOP-induced haptotactic migration of P388D1 cells was completely inhibited by the addition of GRGDS to the upper compartments (Figure 6b).

We next investigated the effects of rOP on haptotaxis induced by FN and VN, and of FN and VN on haptotaxis induced by rOP using Nuclepore filters coated with FN, VN or rOP (2 μg/filter) in the presence of additional fluid-phase FN, VN or rOP respectively, added to the upper compartments at concentrations of 2 μg/ml. Incubation was continued for 4 h at 37 °C, after which time no significant inhibition was observed (Figures 6b, 6c and 6d).

To determine the involvement of different types of integrin receptors, haptotaxis of P388D1 cells to RGD-containing proteins was tested in the presence of anti-integrin mAbs. Haptotaxis of P388D1 cells to rOP was significantly inhibited by anti-α_v mAb, but not by anti-α_4, -α_5 or -α_M mAb, whereas that to FN showed no inhibition with all four mAbs. Haptotaxis of P388D1 cells to VN was significantly inhibited by anti-α_5 and α_v mAbs but not by anti-α_4 and -α_M mAbs. Effects of haptotaxis of P388D1 cells to hOP and profiles of effects of FN, VN and mAbs on haptotaxis of P388D1 cells to hOP were next examined, although the level of haptotaxis of P388D1 cells to hOP is lower than that to rOP (Figure 6a). Haptotaxis of P388D1 cells to hOP was not inhibited in the presence of FN and VN, and was significantly inhibited by anti-α_v but not by anti-α_4, anti-α_5 and

-α_M (Figure 6e). These effects of FN, VN and mAbs on haptotaxis of P388D1 cells to hOP mimic those to rOP.

Migration assay of P388D1 cells for fluid-phase rOP

Chemotactic migration is a phenomenon that describes directional cell migration along a gradient of chemoattractant in the fluid phase, whereas chemokinetic migration describes undirectional random migration. To assess the effects of fluid-phase rOP on cell migration, chemotaxis assays were performed using modified Boyden chambers to determine the migration response of P388D1 cells to various concentrations of rOP, above and below each filter. However, no significant chemotactic migration was observed with rOP (results not shown).

Effects of rOP on leucocyte migration *in vivo*

The ability of rOP to mediate adhesion and haptotaxis may lead to migration of leucocytes *in vivo*. rOP or rOPmut (300 ng) or control PBS was intradermally injected into rabbit skin, and dermal tissue was removed at intervals of 3, 6, 12 and 24 h. Formalin-fixed, paraffin-embedded tissue sections stained with haematoxylin and eosin were investigated for their ability to induce leucocyte migration. Migration of polymorphonuclear leucocytes became evident around venules 3 h after rOP injection, whereas only marginal infiltration was observed with PBS or rOPmut (Figure 7). An anti-rOP affinity column completely adsorbed the reactivity of the rOP preparation. The number of migrating cells around the venules reached a peak 12 h after rOP injection, and subsequently fell to a level comparable with controls by 24 h after injection. No significant migration of monocytes was observed 3–24 h after rOP injection.

DISCUSSION

In the present study, we investigated the expression of rOP and a mutant protein, rOPmut, and determined their abilities to bind P388D1 cells in a saturable fashion (binding affinity, K_d, of 2.35×10^{-10} M) and mediate adhesion and haptotaxis of P388D1 cells.

The primary structure of rabbit OP is highly conserved across five mammalian species with almost perfect preservation of one GRGDS motif which is similar to that of FN [12,14,23,31–34]. The OP sequence of chicken has been reported and shows conservation of the GRGDS sequence [35], although overall homology with rabbit OP is much lower than that between rabbit and other mammalian species. These results strongly suggest that the GRGDS motif plays an important role in OP function. It has been well documented that the GRGDS sequence of FN is involved in cell adhesion through integrin. Osteoclasts have been shown to bind to bone matrix via integrin $\alpha_V\beta_3$ and bone sialoprotein for attachment [28]. In the present study, we have unequivocally demonstrated that the murine macrophage cell line P388D1 is capable of binding to rOP-coated plastic wells in a dose-dependent manner. Several experiments confirmed that the adhesion was mediated by the (G)RGD(S) sequence. First, rOPmut in which glutamate replaced aspartate within the GRGDS sequence showed no adhesion-enhancing ability. Secondly, binding by rOP was completely inhibited by treatment of cells with a synthetic GRGDS peptide. Thirdly, a specific antibody against rOP inhibited rOP-mediated adhesion. Therefore macrophages may bind to matrices via OP by an autocrine mechanism. The present study also showed that rOP is capable of inhibiting the binding effects of FN and vice versa.

The binding of rOP to P388D1 cells was significantly inhibited by both anti-α_4 and -α_5 mAbs, but not by anti-α_V mAb which strongly inhibited the binding of P388D1 cells to VN. Therefore receptors other than $\alpha_V\beta_3$, such as $\alpha_4\beta_1$ and $\alpha_5\beta_1$, probably mediate the binding of rOP to P388D1 cells, although others may be involved as inhibition with anti-α_4 and -α_5 mAbs was relatively weak. FN shows specific affinities for collagen, fibrin, heparin, DNA glycosaminoglycans and cell-surface receptors. Although OP has been suggested to bind positively charged matrix such as hydroxyapatite, its binding activity to other materials has not been investigated. It could therefore paradoxically play a role in the inhibition of macrophage adhesion mediated by FN in tissue other than bone. Inhibition of FN-mediated cell adhesion by an RGD-containing extracellular matrix protein, tenascin, has been reported [36].

The present experiments showed that rOP induces haptotactic migration of P388D1 cells, and experiments using rOPmut, GRGDS peptide and anti-rOP antibody also showed that this phenomenon was mediated via the (G)RGD(S) motif. Receptor use of P388D1 cells for rOP was unexpected because haptotaxis by rOP was not inhibited by anti-α_4 or -α_5 mAb but was markedly inhibited by anti-α_V mAb which had no effect on adhesion of P388D1 cells to rOP. Thus haptotaxis of P388D1 cells induced by rOP could be an α_V-mediated phenomenon. In contrast, rOP induced no directional movement of P388D1 cells. This may be due to the fact that receptors for rOP are different from those for various other chemotactic factors that belong to the superfamily of G-protein-coupled receptors.

Administration of rOP *in vivo* induced significant migration of polymorphonuclear leucocytes around venules as early as 3 h after intradermal injection, and the total number of migrating leucocytes increased with time. In contrast, rOPmut induced only marginal leucocyte infiltration. As most polymorphonuclear leucocytes were scattered over the collagen in the dermis, migration must have occurred mainly around 3 h after rOP injection. The migration of macrophages 3 h and 24 h after intradermal injection of mouse OP separated from the supernatant of a cultured T-cell line, Ar5v, has been reported [27]. However, our experiments showed no significant infiltration of macrophages 3–24 h after injection. This may be due to differences in OP preparation or species differences.

We thank T. Iwao for photography. This work was supported by grants from the Ministry of Education, Science and Culture (no. 06281250), and the Mitsuiseimei Kosei Jigyodan.

REFERENCES

1 Ruoslahti, E. and Pierschbacher, M. D. (1987) Science **238**, 491–497
2 Kornblihtt, A. R., Umezawa, K., Vibe-Pedersen, K. and Baralle, F. E. (1985) EMBO J. **4**, 1755–1759
3 Pierschbacher, M. D. and Ruoslahti, E. (1984) Nature (London) **309**, 30–33
4 Hayman, E. G., Pierschbacher, M. D., Ohgren, Y. and Ruoslahti, E. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 4003–4007
5 Suzuki, S., Oldberg, A., Hayman, E. G., Pierschbacher, M. D. and Ruoslahti, E. (1985) EMBO J. **4**, 2519–2524
6 Plow, E. F., Pierschbacher, M. D., Ruoslahti, E., Marguerie, G. A. and Ginsberg, M. H. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 8057–8061
7 Dedhar, S., Ruoslahti, E. and Pierschbacher, M. D. (1987) J. Cell Biol. **104**, 585–593
8 Lawler, J. and Hynes, R. O. (1986) J. Cell Biol. **103**, 1635–1648
9 Craig, A. M., Nemir, M., Mukherjee, B. B., Chambers, A. F. and Denhardt, D. T. (1988) Biochem. Biophys. Res. Commun. **157**, 166–173
10 Mark, M. P., Prince, C. W., Gay, S., Austin, R. L. and Butler, W. T. (1988) Cell Tissue Res. **251**, 23–30
11 Nomura, S., Wills, A. J., Edwards, D. R., Heath, J. K. and Hogan B. L. M. (1988) J. Cell Biol. **106**, 441–450
12 Smith, J. H. and Denhardt, D. T. (1987) J. Cell. Biochem. **34**, 13–22
13 Yoon, K., Buenaga, R. and Rodan, G. A. (1987) Biochem. Biophys. Res. Commun. **148**, 1129–1136
14 Patarca, R., Freeman, G. J., Singh, R. P. et al. (1989) J. Exp. Med. **170**, 145–161

15 Hynes, R. O. (1992) Cell **69**, 11–25
16 Reinholt, F. P., Hultenby, K., Oldberg, A. and Heinegard, D. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 4473–4475
17 Miyazaki, Y., Setoguchi, M., Yoshida, S., Higuchi, Y., Akizuki, S. and Yamamoto, S. (1989) Nucleic Acids Res. **17**, 3298
18 Miyazaki, Y., Setoguchi, M., Yoshida, S., Higuchi, Y., Akizuki, S. and Yamamoto, S. (1990) J. Biol. Chem. **265**, 14432–14438
19 Sanchez-Madrid, F., Simon, P., Thompson, S. and Springer, T. A. (1983) J. Exp. Med. **158**, 586–602
20 Takada, Y., Strominger, J. L. and Hemler, M. E. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 3239–3243
21 Suzuki, S., Argraves, W. S., Pytela, R., Arai, H., Krusius, T., Pierschbacher, M. D. and Ruoslahti, E. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 8614–8618
22 Maniatis, T., Fritsch, F. F. and Sambrook J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
23 Tezuka, K., Sato, T., Kamikawa, H. et al. (1992) Biochem. Biophys. Res. Commun. **186**, 911–917
24 Studier, F. M., Roseberg, A. H., Dunn, J. J. and Dubendorff, J. W. (1990) Methods Enzymol. **185**, 60–89
25 Nisonoff, A., Wissler, F. C., Lipman, L. N. and Woernley, D. L. (1960) Arch. Biochem. Biophys. **89**, 230–244
26 Fraker, P. J. and Speck, J. C. (1978) Biochem. Biophys. Res. Commun. **80**, 849–857
27 Sigh, R. P., Patarca, R., Schwartz, J., Sigh, P. and Cantor, H. J. (1990) Exp. Med. **171**, 1931–1942
28 Orlando, R. A. and Cheresh, D. A. (1991) J. Biol. Chem. **266**, 19543–19550
29 Yamamoto, S., Yoshinaga, M. and Hayashi, H. (1971) Immunology **20**, 803–808
30 McCarty, J. B., Palm, S. L. and Furcht, L. T. (1983) J. Cell. Biol. **97**, 772–777
31 Oldberg, A., Franzen, A. and Heinegard, D. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 8819–8823
32 Craig, A. M., Smith, J. H. and Denhardt, D. T. (1989) J. Biol. Chem. **264**, 9682–9686
33 Wrana, J. L., Zhang, Q. and Sodek, J. (1989) Nucleic Acids Res. **17**, 10119
34 Kiefer, M. C., Bauer, D. M. and Barr, P. J. (1989) Nucleic Acids Res. **17**, 3306
35 Cstagnola, P., Bet, P., Quarto, R., Gennari, M. and Cancedda, R. (1991) J. Biol. Chem. **266**, 9944–9949
36 Chiquet-Ehrismann, R., Kalla, P., Pearson, C. A., Beck, K. and Chiquet, M. (1988) Cell **53**, 383–390

Received 25 July 1994/22 November 1994; accepted 5 December 1994