

# The Osteoclast Functional Antigen, Implicated in the Regulation of Bone Resorption, Is Biochemically Related to the Vitronectin Receptor

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**Abstract.** We have defined the structure of the Osteoclast Functional Antigen (OFA) by immunological and biochemical means. OFA is an abundant surface antigen in human and animal osteoclasts and has been characterized previously by monoclonal antibodies 13C2 and 23C6, one of which mimicks the inhibitory activity of calcitonin on osteoclastic bone resorption. By the following criteria we show that OFA is a member of the integrin family of extracellular matrix receptors and is identical, or at least highly related, to the vitronectin receptor (VNR) previously isolated from placenta and melanoma cells. Immunoprecipitation analysis demonstrates that OFA from osteoclasts and a monkey kidney cell line Vero is a heterodimeric molecule of 140 kD ( $\alpha$  chain) and 85 kD ( $\beta$  chain) under nonreducing conditions; on reduction at least one low molecular mass ( $\alpha'$ ) species (of  $\sim 30$ -kD size) is released, resulting in a 120/100-kD dimer. Immunoblots of OFA isolated from osteoclasts and Vero cells and VNR purified from placenta and probed with hetero sera to OFA and monoclonal antibodies to platelet gp11a (VNR  $\beta$  chain) show immunological cross-

reactivity between the  $\alpha$  chains of OFA and VNR and the use of gp11a as a  $\beta$  chain by both. OFA from Vero cells binds to an Arg-Gly-Asp containing peptide (GRGDSPPK) isolating a heterodimer recognized by anti-OFA monoclonal antibodies, 13C2 and 23C6. Immunohistochemical analysis showed a similar tissue distribution in humans for the antigen recognized by anti-OFA antibodies, a monoclonal antibody, LM142, raised to melanoma VNR, polyclonal antibodies to the placental VNR and a monoclonal antibody to the presumptive VNR  $\beta$  chain, platelet glycoprotein 11a. Finally, NH<sub>2</sub> terminal amino acid sequencing showed that the amino-terminus of the monkey  $\alpha$  chain was identical in the 12 assigned residues to that of human VNR  $\alpha$  chain. The  $\beta$  chain sequence of OFA differed at least 1 (and up to 4) positions from platelet gp11a (VNR  $\beta$ ) in the first 18 amino acids sequenced. These, and other, data provide the first indication of a function for the VNR and suggest that cell-cell and cell-extracellular matrix interactions involving integrins may play an important role in bone physiology.

**O**STEOCLASTS (OCs)<sup>1</sup> are multinucleate giant cells within bone and are the major cellular effectors of bone resorption (for reviews, see Marks, 1983; Nijweide et al., 1986; Vaes, 1988). A variety of experimental data have shown conclusively that the OC is derived from a bone marrow cell reaching the bone surface via a blood borne mononuclear precursor. These include experimental parabiosis (Göthlin and Ericsson, 1973), quail-chick chimeras (Jotereau and Le Douarin, 1978), the cure by bone marrow transplantation of the abnormality in bone resorption seen in congenital osteopetrosis (Walker, 1975), and the coisolation of OC precursors with those of myeloid colony forming cells (Scheven et al., 1986). Less conclusive is the nature of its

stem cell and route and control of its differentiation. Thus a major point of contention has been over the exact relationship between the OC and cells of myeloid lineage (discussed in Nijweide et al., 1986; Horton, 1988). Since OCs are known to be formed by cell fusion, they have conventionally been considered to be a specialized form of skeletal macrophage, analogous to the inflammatory macrophage polykaryon (Chambers, 1978). However, although they share a number of properties, they differ in several important ways. For example, terminally differentiated macrophages do not have calcitonin receptors (Nicholson et al., 1986) nor do they respond to calcitonin (Chambers and Magnus, 1982), and they are incapable of resorbing inert bone in vitro (Chambers et al., 1984; Chambers and Horton, 1984), all of which are functional hallmarks of the OC. These differences do not, however, preclude a common ancestry since terminal differ-

1. *Abbreviations used in this paper:* OC, osteoclast; OFA, osteoclast functional antigen; OGP, octyl glycopyranoside; VLA, very late antigen; VNR, vitronectin receptor.

entiation in the environment of bone could conceivably account for such disparities. Thus, the stage at which OCs and other haemopoietic cells are related, whether only distantly as suggested by some transplantation (Loutit and Nesbit, 1982) and phenotypic (Horton et al., 1984, 1985a) studies or more closely at the stage of an immature monocyte (Burger et al., 1982), is not known.

In an attempt to resolve this question, several groups including our own (Horton et al., 1985b; Oursler et al., 1985; Nijweide et al., 1985) have produced mAbs specific for OC antigens for use in the analysis of OC differentiation. Antibodies 13C2 and 23C6 (Horton et al., 1985b) were raised to a human tumour, the giant cell tumor of bone or osteoclastoma, which is uniquely enriched with OCs (Fig. 1). These two mAbs were shown subsequently to have a high degree of specificity for human OCs (Horton et al., 1985b), generally failing to react with other haemopoietic or bone cells including those of the mononuclear phagocyte system; to be widely expressed on nonhuman mammalian and avian OCs (Horton and Chambers, 1986); to be developmentally regulated during embryogenesis, appearing early on preosteoclasts adjacent to bone anlagen (Simpson and Horton, 1989); and to mediate inhibitory effects on osteoclastic bone resorption *in vitro* (Chambers et al., 1986), suggesting a functional role in bone for the molecule that the mAbs recognize.

In this paper we describe the characterization of the molecule recognized by mAbs 13C2 and 23C6, the osteoclast functional antigen (OFA) (Davies et al., 1988) and demonstrate that it is a member of the family of tissue-specific extracellular matrix receptors (Ruoslahti and Pierschbacher, 1987) termed integrins (Hynes, 1987), showing a high degree of homology, or identity, to the vitronectin receptor (Pytela et al., 1985b; Suzuki et al., 1986 and 1987; Fitzgerald et al., 1987b). OFA thus forms a hitherto unrecognized class of receptor on OCs that is likely to have functional importance in the regulation of bone resorption.

## Materials and Methods

### Cell Lines and Tissues

The Vero monkey kidney cell line (Fig. 1 *a*) was maintained in log phase growth by twice weekly passage and growth in RPMI 1640 (CRPMI) medium containing 10% FCS, glutamine, and antibiotics.

Platelets were prepared from whole blood of normal volunteers, anticoagulated with Acid-Citrate-Dextrose (ACD) solution and isolated by differential centrifugation.

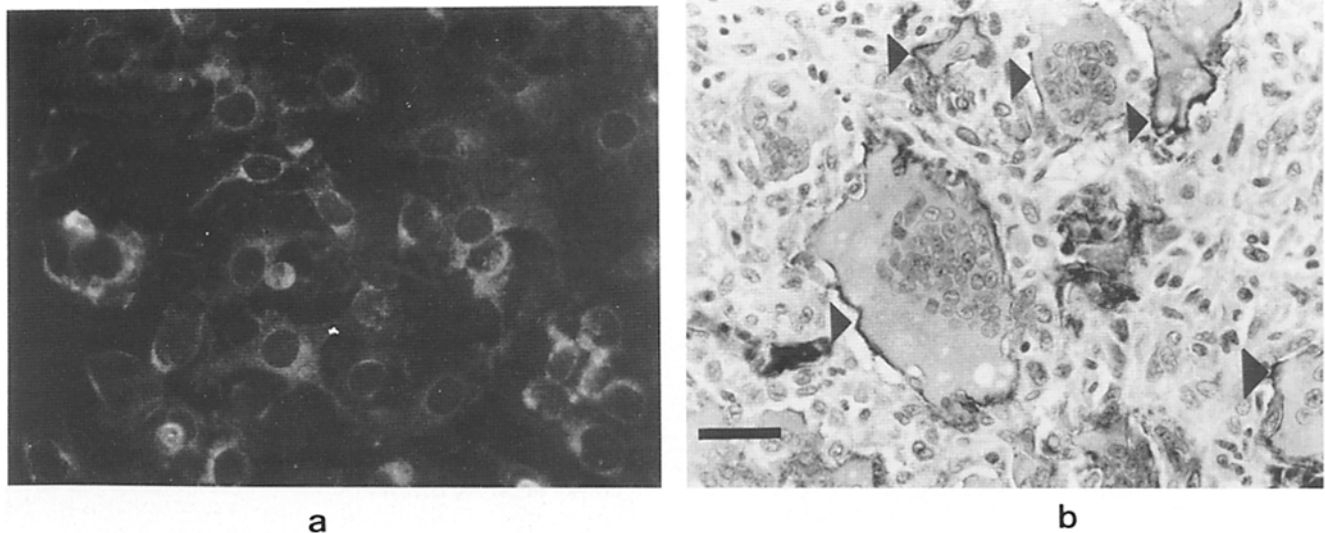
Tissues for immunohistology or biochemical analysis were obtained from surgical removal (samples of giant cell tumor of bone [Fig. 1 *b*]), parturition (full term placental), postmortem (kidney), or following therapeutic abortion (fetal bone). All tissues were obtained with ethical committee approval, and, in the case of fetal material, the recommendations of the Peel Report were followed. Tissues for biochemical studies were snap frozen and stored dry in liquid nitrogen until use. Tissues for immunohistology were snap frozen in OCT (TissueTec Laboratories Inc., IN) embedding medium and stored in liquid nitrogen until use. Human fetal bones were dissected from 12–16-wk fetuses, cleaned of excess connective tissue, chopped, and tissue imprints prepared as previously described (Horton et al., 1985a,c); these were subsequently air dried, fixed in dry acetone for 10 min and stored frozen at  $-20^{\circ}\text{C}$  for use within 1 mo of preparation.

### Antibodies

Monoclonal antibodies to giant cell tumour of bone, 13C2 and 23C6, as described (Horton et al., 1985b) were used as tissue culture supernatants throughout the study. Some features of their immunohistological (Horton et al., 1985b; Warwick et al., 1987) and functional reactivity (Chambers et al., 1986) have been reported previously. Antibody 5/9 (Horton et al., 1985b) was used as an IgG1 isotype control for experiments using monoclonals 13C2 and 23C6.

Monoclonal antibodies to platelet gp11a (V1-PL12; Dr. Knapp, University of Vienna, Vienna) and gp11b/11a complex (P256; Dr. Hogg, I. C. R. F., London) were gifts of their originators and have previously been characterized in an international workshop (Hogg and Horton, 1987).

Monoclonals LM142 and LM609 (gift of Dr. Cheresch, Research Institute of Scripps Clinic, La Jolla, CA) were raised against the M21 melanoma cell line and characterized as reacting with the vitronectin receptor (VNR) (Cheresch and Harper, 1987; Cheresch and Spiro, 1987). A polyclonal rabbit antiserum raised against Arg-Gly-Asp peptide affinity-purified human placental VNR (Suzuki et al., 1986) was kindly provided by Dr. Ruoslahti (La



**Figure 1.** (a) Immunofluorescence photomicrograph of acetone-fixed Vero monkey kidney cells stained with mAb 23C6 showing membrane and cytoplasmic distribution of OFA. (b) Wax-embedded section of formalin-fixed osteoclastoma (giant cell tumor of bone) stained by indirect immunoperoxidase technique with rat polyclonal antiserum to immunoaffinity purified OFA heterodimer. Note that the large multinucleate OCs show a surface membrane distribution of product (arrowhead) whereas the majority of mononuclear cells (stromal cells and infiltrating leucocytes) are negative. Bar, 100  $\mu\text{m}$ .

Jolla Cancer Research Foundation, La Jolla, CA) or purchased from Telios Pharmaceuticals Inc. (San Diego, CA).

Rat and rabbit polyclonal antibodies were prepared to the OFA antigen, purified as below (Krissansen et al., 1986). The rabbit and rat (named FRI) heterosera showed biochemical and immunological characteristics similar to mAbs 13C2 and 23C6 and the polyclonal antipalcental VNR sera; they were used without further purification.

### Immunohistology

Immunostaining of frozen sections of human tissues (giant cell tumour of bone, placenta, and kidney), cytospin preparations of Vero monkey kidney cells, or human fetal bone imprints, was performed on acetone-fixed material using the indirect immunofluorescence or immunoperoxidase techniques as previously described (Horton et al., 1984, 1985b).

### Purified Proteins

VNR purified by RGD peptide affinity chromatography (Pytela et al., 1987) was a kind gift of Dr. E. Ruoslahti.

### Cell and Protein Labeling Techniques

Confluent cultures of  $10^7$  Vero cells were metabolically labeled by incubation for 2 h with 0.2 Ci/ml [ $^{35}$ S]methionine (Amersham International, Amersham, UK) in methionine-free medium and chased for 68 h with methionine-replete RPMI 1640 growth medium containing 10% FCS.

A cell suspension of giant cell tumor of bone containing a high proportion of OCs was labeled by lactoperoxidase catalysed  $^{125}$ I-iodination (IMS-30, Amersham International) (Hubbard and Cohn, 1972). FPLC size-fractionated, partially purified OFA dimer (see below) was iodinated by the chloramine T method (Hunter and Greenwood, 1962).

Metabolically and surface iodinated cells were solubilized by lysis on ice in NP-40 buffer (20 mM Hepes, pH 7.2, 50 mM NaCl, 0.3 M sucrose, 3 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.5% NP40, 1 mM PMSF), nuclear and cellular debris removed by centrifugation and the lysate precleared by the addition of 50  $\mu$ l of a 10% solution of formalin-fixed *Staphylococcus aureus* Cowan A strain. Primary antibodies (13C2, 23C6, 5/9, P256; 200  $\mu$ l culture supernatant or 10  $\mu$ l ascitic fluid) were coincubated for 90 min on ice before isolation of the antibody-antigen complexes with *S. aureus* precoated with rabbit anti-mouse IgG serum (Dako Corp., Santa Barbara, CA). The *S. aureus* pellet was retrieved by centrifugation and washed thrice in lysis buffer to reduce background contamination. Iodinated (mAb or peptide affinity) purified OFA  $\alpha/\beta$  heteroimer was treated similarly. Analysis of the immunoprecipitates was by SDS-PAGE (Laemmli, 1970) under nonreducing or reducing (5% vol/vol 2-mercaptoethanol) conditions followed by autoradiography; [ $^{35}$ S]methionine gels were subjected to fluorography (Bonner and Laskey, 1974).

### Western blotting

Lysates of giant cell tumor of bone, platelets and Vero cells; immunoaffinity-purified OFA and RGD-purified placental VNR (Pytela et al., 1987); 13C2/23C6 or P256 immunoprecipitates of platelets and Vero cells were electrophoresed in 7.5% SDS-PAGE under reducing conditions and the proteins transferred onto 0.45- $\mu$ m pore size nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH) by semi-dry electroblotting (200 mA, 1 h, using Sartorius apparatus and manufacturer's stated conditions). Completion of transfer was monitored by silver staining of the gel (Morrissey, 1981) and transfer of Coomassie blue-stained molecular mass markers (Sigma Chemical Co., St. Louis, MO). Nonspecific binding to the membranes was blocked by preincubation with 1% gelatin (Sigma Chemical Co.). Antigen was localized by incubation with rat (FRI) or rabbit anti-OFA; bound antibody was visualized by sequential incubation with biotinylated anti-species Ig reagents, peroxidase-conjugated avidin with color development using 4-chloro-1-naphthol.

### Peptide Affinity Chromatography of the Vero Receptor

Vero receptor was isolated according to previously established techniques (Pytela et al., 1985a,b).  $5 \times 10^7$  surface-iodinated Vero cells were lysed at 4°C in 1 ml buffer (PBS pH 7.4 containing 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 200 mM octylglycopyranoside [OGP], and 3 mM PMSF). The lysate was centrifuged to remove cellular debris, incubated overnight at 4°C with 1 ml of GRGDSPPK-Sepharose; the peptide column was made by reacting 20 mg acetylated GRGDSPPK peptide (kindly provided by Dr. J. Rothbard,

I.C.R.F., London) under standard conditions with 1 ml of CNBr-Sepharose (Sigma Chemical Co.). The column was then washed with 5 ml PBS containing 50 mM OGP, 1 mM PMSF followed by 2 ml of buffer containing 1 mg/ml free GRGDSPPK peptide. Fractions of 0.5 ml were collected and the ethanol-precipitated protein from 100- $\mu$ l aliquots of each fraction were analyzed by electrophoresis on 11% SDS-polyacrylamide gels under reducing conditions.

### OFA $\alpha$ and $\beta$ Chain Purification and NH<sub>2</sub>-terminal Amino Acid Sequencing

The OFA dimer was purified (Krissansen et al., 1986) from Vero cells using mAb affinity chromatography with both mAbs 13C2 and 23C6, linked to Sepharose CL4B (Pharmacia Fine Chemicals, Uppsala) at pH 7.0 using the method of Kumel et al. (1979). The two monoclonals, which recognize different epitopes on the same molecule, were combined as they were found to markedly increase antigen yield in preparative affinity chromatography when used together. The isolated proteins were electrophoresed in a non-reduced 7.5% polyacrylamide/SDS gel with 1 mM thioglycollate added to the upper buffer reservoir. The separated  $\alpha$  and  $\beta$  chains of OFA were visualized by exposure to 4 M Na acetate (Higgins and Dahmus, 1979) cut from the gel and passively eluted into 50 mM ammonium bicarbonate pH 8.0, 0.1% SDS, 0.1 mM DTT buffer. The proteins were then reduced and alkylated with 10 mM DTT/22 mM iodoacetamide (Waxdal et al., 1968) and recovered by precipitation with 10% trichloroacetic acid. The  $\alpha$  chain was dissolved in 6 M guanidine HCl in 0.1 M KH<sub>2</sub>PO<sub>4</sub>, pH 4.5, purified by FPLC on two Superose 12 columns in series eluted with 70% formic acid. Two samples of 9  $\mu$ g of each protein, as determined by amino acid analysis (6300 analyser; Beckman Instruments, Palo Alto, CA) were subjected to Edman degradation using a sequencer (477A; Applied Biosystems, Inc., Foster, CA) equipped with a 120 A on line phenylthiohydantoin amino acid analyzer.

### Results

#### Immunoprecipitation Analysis of the Antigen Recognized by Anti-OFA Monoclonal Antibodies, 13C2 and 23C6, in OC and the Vero Cell Line

Monoclonal antibody 23C6 immunoprecipitates a heterodimeric molecule (Fig. 2, lane 4) from human OC derived

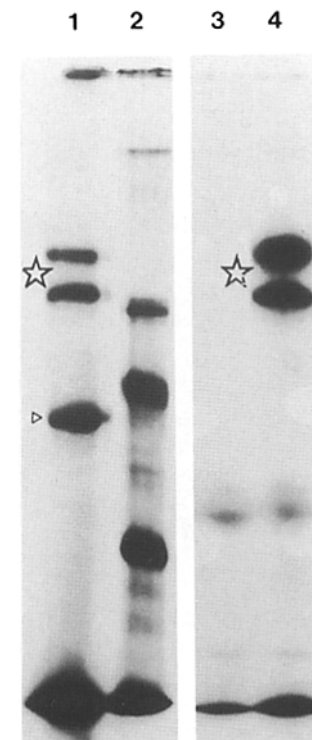
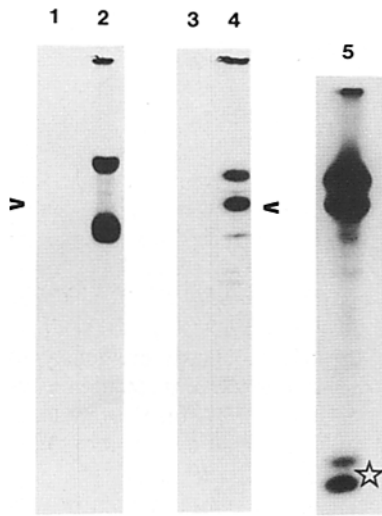


Figure 2. Immunoprecipitation analysis, with mAbs 13C2 and 23C6, of human OCs and the monkey cell line Vero. Lane 1, chloramine T iodinated OFA antigen (star) immunoaffinity purified with 13C2 and 23C6 mAbs from the Vero kidney cell line. BSA was used to quench the iodination reaction and is indicated (arrowhead). Lane 2, molecular mass markers (from top to bottom, 200, 92.5, 69, 46, and 30 kD). Lanes 3 and 4, immunoprecipitation analysis of iodinated osteoclasts from a giant cell tumor of bone, using mAb 23C6 (star) (lane 4) or negative control antibody, P256, to platelet gp1b/111a complex (lane 3). All samples were reduced and subjected to 10% SDS-PAGE.



**Figure 3.** Characteristics of OFA from the Vero cell line under non-reducing and reducing conditions. Lanes 1 and 3, negative control immunoprecipitations using mAb 5/9 of the same IgG1 isotype. Lanes 2 and 4, 13C2 (or 23C6, not shown) mAb immunoprecipitated OFA from [<sup>35</sup>S]methionine metabolically labeled Vero cells run under nonreducing (lane 2) and reducing (lane 4) conditions. Lane 5, FPLC size-fractionated Vero antigen was prepared under nonreducing conditions, iodinated, immunoprecipitated with anti-OFA antibody, and analyzed by SDS-PAGE under reducing conditions. Minor protein species released from OFA upon reduction (*star*). All proteins were analyzed by 10% SDS-PAGE; the position of the 92.5 kD molecular mass marker is indicated (*caret*).

from giant cell tumor of bone (Fig. 1 *b*). Similar results (not shown, but see Fig. 3) were obtained with 13C2 monoclonal. As predicted from immunohistology, (Fig. 1 *a*), a similar sized dimer is isolated from the Vero monkey kidney cell line

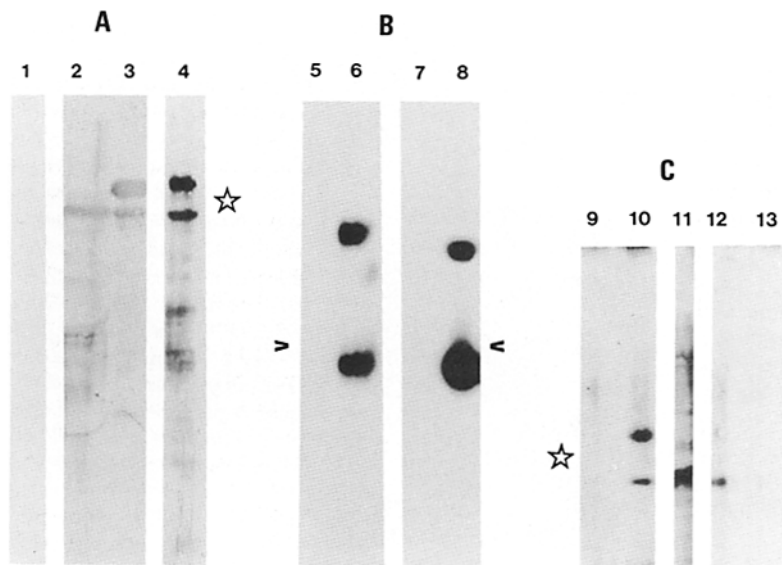
(and the ACHN human renal line, not shown), which showed high levels of OFA expression (Fig. 2, lane 1) using the same antibodies. Under reducing conditions the molecular sizes of the two chains isolated from OC and kidney are of 120 kD and 100 kD apparent molecular mass. The terminology used in this paper calls the higher molecular mass species the  $\alpha$  chain and the lower the  $\beta$  chain of OFA.

Because of the difficulty in obtaining large numbers of OC, even from bone tumors, the Vero monkey kidney cell line was used as the source of antigen for the primary biochemical and immunochemical characterization of OFA.

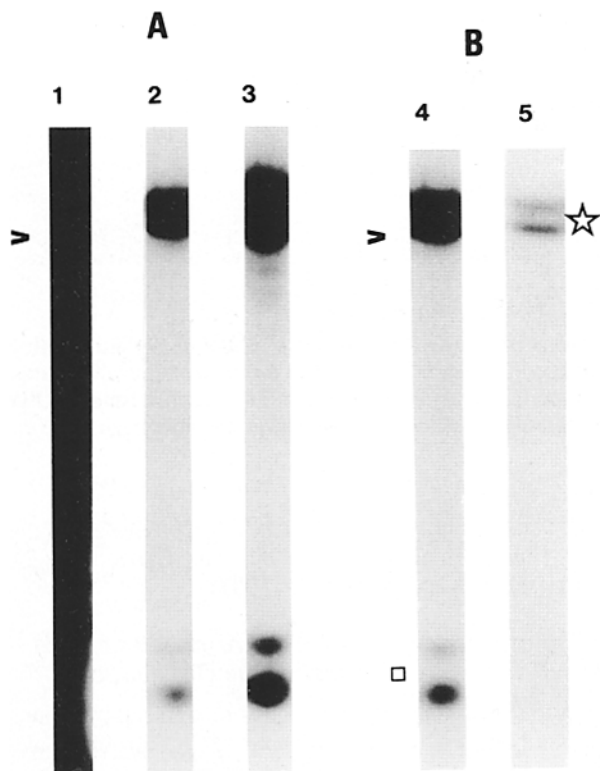
A particular feature of the OFA molecule is its behavior on reduction. Fig. 3 (lane 2) shows that the mAbs 13C2 (or 23C6) recognize an  $\alpha$  chain of 140 kD and  $\beta$  chain of 85 kD in the Vero cell line when electrophoretically separated under nonreducing conditions. Upon reduction (Fig. 3, lane 4) the molecular mass of the  $\alpha$  chain falls to 120 kD and the  $\beta$  chain increases to 100 kD. Reduction is accompanied by the release of low molecular mass peptides ( $\alpha'$  chain) of  $\sim$ 30-kD size. This is shown in Fig. 3 (lane 5) where the OFA  $\alpha$  and  $\beta$  chain complex has been immunopurified under non-reducing conditions, size fractionated by FPLC and finally separated electrophoretically under reducing conditions. Shifts in size upon reduction are characteristic of members of the integrin receptor family; for example, platelet gp1b/11a complex and the vitronectin and fibronectin receptors (Pytela et al., 1986).

#### **Demonstration that the $\beta$ chain of OFA Is Immunologically Cross-reactive with "Platelet" gp11a**

Previous immunohistological studies (Horton, 1986; Athanasou et al., 1986) have demonstrated that OCs express gp11a, but not other platelet-specific membrane proteins. Molecular cloning (Fitzgerald et al., 1987a; Rosa et al., 1988) and im-



**Figure 4.** Demonstration of immunological identity of the  $\beta$  chain of OFA and platelet gp11a. *A*, Immunoblot analysis of whole platelet lysate (lane 2), Vero surface membranes (lane 3), and immunoaffinity-purified Vero antigen (lane 4). Proteins were electrophoresed through 7.5% polyacrylamide gel under reducing conditions, electrophoretically transferred onto nitrocellulose paper and probed with normal rat serum (lane 1) or a rat heteroserum (FRI) raised against purified OFA that reacts with both  $\alpha$  and  $\beta$  chains of OFA. Bound antibody was detected using biotinylated anti-rat-IgG and horseradish peroxidase conjugated avidin. Positions of  $\alpha$  and  $\beta$  bands (*star*). *B*, Immunoprecipitation of surface-iodinated Vero cells (lanes 5 and 6) and platelets (lanes 7 and 8) with antiplatelet gp11b/11a, P256, (lanes 5 and 8) and anti-OFA mAbs (lanes 6 and 7) run out on SDS-PAGE under nonreducing conditions in the presence of EDTA. The position of the 92.5-kD molecular mass marker is indicated (*caret*). *C*, immunoblot analysis using rat antiserum, FRI, of immunoprecipitates from Vero (lanes 9 and 10, 12 and 13), prepared as in *B*, compared with whole platelet lysate (lane 11), electrophoresed under reducing conditions in SDS-polyacrylamide gels. Positions of  $\alpha$  and  $\beta$  bands (*star*).



**Figure 5.** Affinity chromatography of OGP extracts of surface-iodinated Vero cells on GRGDSPPK-Sepharose. Surface-iodinated Vero cells were lysed in extraction buffer, incubated overnight at 4°C with 1 ml of GRGDSPPK-Sepharose. The column was then washed with PBS containing 50 mM OGP, 1 mM PMSF, followed by 2 ml of column buffer containing 1 mg/ml of soluble GRGDSPPK peptide. 0.5-ml fractions were collected, and the ethanol-precipitated protein from 100- $\mu$ l aliquots of each fraction was analyzed on 11% SDS-PAGE under reducing conditions. The tracks represent the following elution fractions: *A*, lane 1, Vero lysate applied to column; lane 2, column wash; lane 3, 1 mg/ml GRGDSPPK eluate. *B*, lanes 4 and 5, immunoprecipitation of eluted material with antibody 13C2 (or 23C6 not shown) exposed for different times to show the  $\alpha/\beta$  dimer (*star*) and low molecular mass cleaved peptides (*square*). The position of the 92.5-kD molecular mass marker is indicated (*caret*).

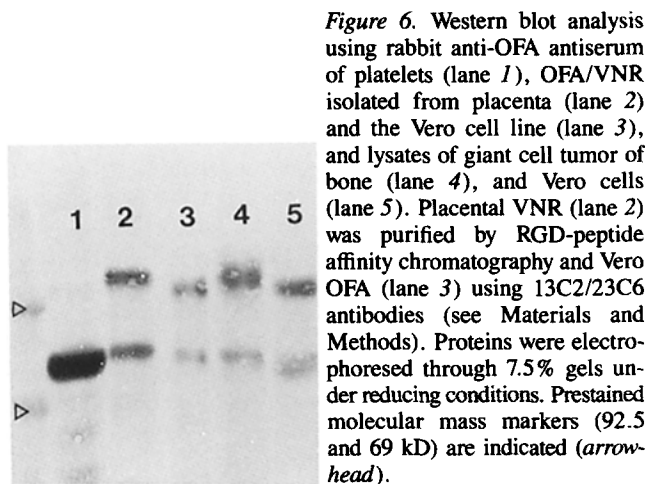
munochemical studies (Cheresh and Harper, 1987; Ginsberg et al., 1987) of the VNR have also shown that the  $\beta$  chain of VNR is platelet gp11a. The similarity in size of the OFA  $\beta$  chain and gp11a led us to investigate whether they were identical. Western blots of platelet and Vero cell lysates using a rat heteroserum (FRI) made to purified OFA  $\alpha/\beta$  chains (Fig. 4 *A*) (which detects OCs immunohistologically [Fig. 1 *b*]) suggested that the two were immunologically related. This was proven by Western blotting immunoprecipitates of platelet gp11b/11a and Vero-derived OFA  $\alpha/\beta$  heterodimer (Fig. 4 *B* and *C*) with the heteroserum. The gp11a chain of platelet gp11b/11a (isolated from platelets with an mAb P256, to gp11b/11a, Fig. 4 *B*, lane 8) was recognized by FRI antiserum (Fig. 4 *C*, lane 12) as was the OFA  $\alpha$  (and  $\beta$ ) chain from the Vero cell line (Fig. 4 *C*, lane 10) (isolated by precipitation with 13C2 and 23C6 mAbs, Fig. 4 *B*, lane 6).

### OFA Binds to an Octapeptide Affinity Matrix That Contains the Arg-Gly-Asp Fibronectin Recognition Sequence

The behavior of OFA on reduction and the immunological similarity of the OFA  $\beta$  chain to gp11a led us to postulate that it formed part of the family of membrane receptors that recognize extracellular matrix molecules containing the Arg-Gly-Asp (RGD) amino acid sequence, of which the platelet gp11a/11a complex (Pytela et al., 1986) and VNR (Pytela et al., 1985*b*) are well-characterized examples. We analysed the binding of OFA antigen from Vero to an immunobilized synthetic octapeptide, GRGDSPPK, shown previously to bind to many of the family of cytoadhesive molecule receptors (Ruoslahti and Pierschbacher, 1987) (Fig. 5). A molecular complex, identical in size and chain structure to OFA, bound to the peptide column and was eluted by free GRGDSPPK peptide (Fig. 5, lane 3) but not with control GRGESPPK peptide; the eluate was shown to be immunologically identical to OFA by immunoprecipitation with mAbs 13C2 (and 23C6, not shown) (Fig. 5, lanes 4 and 5). Thus OFA, like the VNR and platelet gp11b/11a, recognizes Arg-Gly-Asp-containing peptide ligands (Ruoslahti and Pierschbacher, 1986 and 1987).

### Immunological Identity of OFA and Arg-Gly-Asp Peptide Affinity-purified Placental VNR: Western Blot Analysis with Polyclonal Antisera to OFA

Polyclonal antisera made to 23C6/13C2 mAb-purified Vero OFA detect both the  $\alpha$  and  $\beta$  chains of OFA purified from Vero cells (i.e., the immunogen; Fig. 6, lane 3) and VNR purified from placenta by RGD peptide affinity chromatography (Pytela et al., 1987) (Fig. 6, lane 2); these data support the identity, or at least immunological similarity, of the two receptor molecules. The same sera react with a dimer of similar molecular mass derived directly from osteoclastoma tumor (Fig. 6, lane 4) and Vero cells (Fig. 6, lane 5). The minor differences in the mobility of the OFA bands seen in Fig. 6 were not consistent between experiments. Finally, and compatible with the  $\beta$  chain of OFA being platelet gp11a (see earlier section), anti-OFA sera react with the  $\beta$  chain (gp11a) of the platelet integrin, gp11b/11a (lane 1).



**Figure 6.** Western blot analysis using rabbit anti-OFA antiserum of platelets (lane 1), OFA/VNR isolated from placenta (lane 2) and the Vero cell line (lane 3), and lysates of giant cell tumor of bone (lane 4), and Vero cells (lane 5). Placental VNR (lane 2) was purified by RGD-peptide affinity chromatography and Vero OFA (lane 3) using 13C2/23C6 antibodies (see Materials and Methods). Proteins were electrophoresed through 7.5% gels under reducing conditions. Prestained molecular mass markers (92.5 and 69 kD) are indicated (*arrowhead*).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
OFA $\alpha$ chain (monkey) <sup>a</sup>	Phe	Asn	Leu	Asp	Val	Asp	Ser	Pro	Ala	Glu	Tyr	?	Gly					
VNR $\alpha$ chain (human)	Phe	Ash	Leu	Asp	Val	Asp	Ser	Pro	Ala	Glu	Tyr	Ser	Gly					
OFA $\beta$ chain (monkey) <sup>b</sup>	Gly	Pro	Asn	Ile	Cys	Thr	(Gly/Thr)	Leu*	Gly	Val	Ser	Leu <sup>‡</sup>	?	Gln	?	Gln <sup>‡</sup>	Leu	Ala
VNR $\beta$ chain (human)	Gly	Pro	Asn	Ile	Cys	Thr	Thr	Arg	Gly	Val	Ser	Ser	Cys	Gln	Gln	Cys	Leu	Ala

a. Structural homology with the NH<sub>2</sub> termini of OFA/VNR $\alpha$  has been observed for gp11b (Charo et al., 1986; Fitzgerald et al., 1987b; Poncz et al., 1987) LFA-1/CD11a (Springer et al., 1985); Mac-1/CD11b (Arnaout et al., 1988); p150/CD11c (Corbi et al., 1987); VLA $\alpha$  chains (Takada et al., 1987); fibronectin receptor/VLA-5 (Argaves et al., 1986).

b. Lack of homology has been noted between OFA/VNR $\beta$  (gp111a) and chicken integrin Band 3 (VLA $\beta$ ) (Tamkun et al., 1986) and LFA  $\beta$  (CD18) (Kishimoto et al., 1987).

**Figure 7.** Amino-terminal amino acid sequencing of  $\alpha$  and  $\beta$  chains of OFA. The amino-terminal amino acid sequence of the Vero monkey  $\alpha$  (120 kD) (a) and  $\beta$  (100 kD) (b) chains of OFA, as determined in this study, are shown in comparison with the amino-terminal sequence of the human VNR (Charo et al., 1986; Suzuki et al., 1987; Fitzgerald et al., 1987a,b; Rosa et al., 1988). \*,  $\beta$ 8 difference between OFA and VNR; ?, unclear PTH signal; ( ), OFA  $\beta$ 7 signal Gly or Thr; †, OFA  $\beta$ 12 and 16 amino acid assignment probable.

### Amino Terminal Sequencing of the $\alpha$ and $\beta$ Chains of OFA Purified from the Vero Monkey Kidney Cell Line Reveals Homology with the Human VNR

We have isolated the two high molecular mass chains of OFA from Vero cells, analyzed their amino acid composition and sequenced their NH<sub>2</sub>-terminal amino acids (Fig. 7). Because of the scarcity of OC-rich giant cell tumor of bone, we did not have the opportunity to confirm this sequence data from (presumed) OCs; the immunological and biochemical data presented herein would support our use of Vero cells as a reasonable alternative source of OFA for sequencing.

Sequence from the OFA  $\beta$  chain shows a number of differences (at least one, position 8, and up to four, positions 7, 12, and 16 additionally; see Fig. 7) from the published sequences of platelet gp11a from platelets, endothelium and HEL erythroleukaemia cells (Charo et al., 1986; Fitzgerald et al., 1987a; Rosa et al., 1988). Taken with its high cysteine content (not shown), this data supports our immunological and biochemical findings (Figs. 4 and 8) that the  $\beta$  chain of OFA was likely to be gp11a.

The sequence of the OFA  $\alpha$  chain (Fig. 7) is essentially identical to that of the VNR  $\alpha$  chain (Suzuki et al., 1986 and 1987; Fitzgerald et al., 1987b) with an uncertain amino acid designation at position 12 for OFA. In general, integrin receptor  $\alpha$  chain NH<sub>2</sub>-terminal sequences are homologous though distinctive, particularly within the subgroups of the supergene family (compared in Takada et al., 1987). This contrasts with some nonintegrin adhesion receptors, for example the cadherins, which are identical at their NH<sub>2</sub> termini (Shirayoshi et al., 1986). These results thus strongly suggest that the OFA and VNR  $\alpha$  chains are one and the same.

A computer search has confirmed the relationship between the OFA/VNR  $\alpha$  chain and members of the Arg-Gly-Asp (RGD) cytoadhesive molecule receptor family that has been noted by others: platelet gp11b (Charo et al., 1986; Poncz et al., 1987; Fitzgerald et al., 1987b), LFA-1/CD11a (Springer et al., 1985), Mac-1/CD11b (Arnaout et al., 1988), p150/CD11c (Corbi et al., 1987) and the very late antigen (VLA)  $\alpha$  chains (Takada et al., 1987), including that of the fibronectin receptor/VLA-5 (Argaves et al., 1986) showing similarities to OFA at their NH<sub>2</sub> termini.

The OFA  $\beta$  chain showed a high degree of homology to gp11a (Fig. 7), but not to the  $\beta$  chains of the other integrin

families: chicken integrin band 3 (the avian equivalent of the VLA  $\beta$  chain) (Tamkun et al., 1986) and CD18, the LFA family  $\beta$  chain (Kishimoto et al., 1987).

### Immunohistochemical Analysis of Antibodies to OFA and VNR Reveal an Identical Tissue Distribution

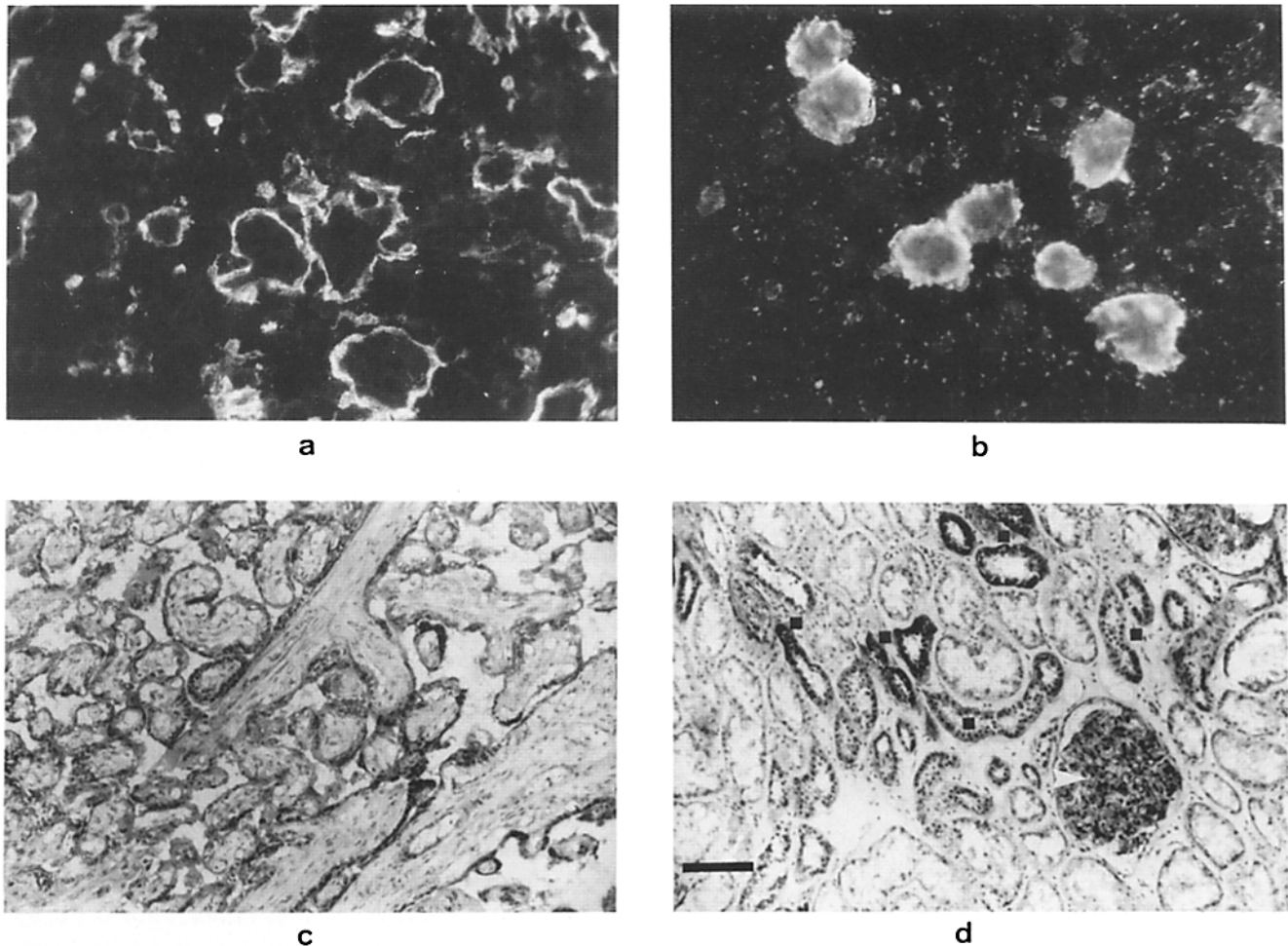
The tissue distribution of the antigens recognized by monoclonal antibodies to OFA (13C2 and 23C6, Horton et al., 1985b) and melanoma VNR (LM142, Cheresch and Harper, 1987; Cheresch and Spiro, 1987) is summarized in Table I and illustrated in Fig. 8. In vivo, both sets of antibodies bind strongly to OCs in bone tumors and fetal bone, placenta, and kidney (glomerulus and distal tubule) and weakly to vascular endothelium (not shown); they fail to react with osteoblasts and bone marrow cells. A similar immunohistochemical distribution is seen in the rabbit and chicken (mAb 23C6 only, Horton and Chambers, 1986). Polyclonal antibodies to purified OFA raised in rat and rabbit (observations in this paper) show a similar tissue specificity to mAbs 13C2 and 23C6 (data not shown; Warwick, J. and M. Horton, manuscript in preparation).

Heterosera to RGD-purified placental VNR ( $\alpha$  and  $\beta$  chain-reactive) (Suzuki et al., 1986) also show a similar reaction to monoclonal antibodies to OFA (Table I).

In comparison, mAb VI-PL2 (Hogg and Horton, 1987), which was raised against platelets and detects platelet gp11a (OFA/VNR  $\beta$  chain, see above) reacts with a similar range of cell types as antibodies to OFA/VNR (Table I). Additionally, strong reactivity with cells of the platelet-megakaryocytic lineage is seen, presumably because of detection of the other heterodimer that uses gp11a as its  $\beta$  chain-platelet gp11b/111a. Minor differences are, however, seen in vivo (Table I) including stronger reactivity of VI-PL2 with vascular endothelium, and weak or absent staining of renal tubules and placenta. The basis for the variation in tissue distribution is at present unclear.

Although expression of the OFA/VNR is quite restricted in vivo, its distribution is much more widespread if cultured adherent cell lines are considered. An extensive comparison of its expression on cell lines is outside the scope of this paper; however, it is worth commenting that though OFA/VNR is "absent" from osteoblasts in vivo, many in vitro osteosarcoma (hence osteoblast-derived) cell lines express moderate levels of OFA/VNR (summarized in Table I). Indeed, the





**Figure 8.** Immunohistological staining of OFA-expressing human tissues by the immunofluorescence and immunoperoxidase techniques. All use the mAb 23C6, but equivalent results were obtained with mAbs 13C2 (Horton et al., 1985b) and LM142 (Cheresh et al., 1987). (a) Frozen section of OC-containing giant cell tumor of bone. Membrane immunofluorescence staining is seen on large multinucleate and some mononuclear cells (b). Immunofluorescence staining of a tissue imprint from a 14-wk gestation human fetal long bone showing positive staining of OCs. Note the absence of staining of other marrow or bone elements in this preparation. (c) Frozen section of human full-term placenta showing strong immunoperoxidase reaction in the syncytiotrophoblast layer. (d) Frozen section of human kidney showing weak reaction in glomeruli (white arrowhead), and stronger reaction with distal renal tubules (black square). Bar, 100  $\mu$ m.

VNR  $\alpha$  chain was first purified from the MG63 osteosarcoma cell line (Pytela et al., 1985b).

### Discussion

In this paper we describe the characterization of a cell surface (glyco)protein, OFA (Davies et al., 1988), which is abundant in OCs and otherwise limited in its distribution *in vivo* to kidney and placenta. In particular, its lack of expression by cells of the mononuclear phagocyte system is noteworthy (Horton et al., 1985b). Several lines of evidence, both biochemical and immunological, point to OFA belonging to the family of extracellular matrix receptors termed variously integrins or cytoadhesins (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Ginsberg et al., 1988). First, OFA consists of an  $\alpha/\beta$  heterodimeric (glyco)protein complex (Fig. 2) with a low molecular mass disulphide bond linked  $\alpha'$  chain (or possibly two chains) (Figs. 2 and 3) released on reduction. Second, the complex binds to a fibronectin con-

sensus sequence-derived (Pierschbacher and Ruoslahti, 1984) Arg-Gly-Asp (RGD)-containing affinity matrix and can be eluted with cognate peptide (Fig. 5). Third, Western blots of heterodimers isolated from OCs, the Vero cell line, placenta, and platelets show that the  $\beta$  chain of OFA is immunologically cross-reactive with platelet gp11a and the  $\alpha$  chain with the  $\alpha$  chain of VNR (Figs. 4 and 6). Fourth, NH<sub>2</sub>-terminal amino-acid sequencing of OFA  $\alpha$  and  $\beta$  chains (Fig. 7), purified from the Vero primate kidney cell line, shows homology/identity ( $\alpha$  chain) or a high degree of homology ( $\beta$  chain) at the amino acid level with the NH<sub>2</sub> termini of the VNR  $\alpha$  chain (for OFA  $\alpha$ ) and platelet gp11a (for OFA  $\beta$ ). Differences in NH<sub>2</sub>-terminal amino-acid sequence of the  $\beta$  chain may be because of species differences, the OFA sequence being of primate (Vero monkey) origin whereas the published sequence of VNR  $\beta$  (Fitzgerald et al., 1987a; Rosa et al., 1988) comes from studies of human tissue; however, the possibility of a second gp11a-like  $\beta$  chain cannot be excluded. Finally, the tissue distribution *in vivo* of OFA and

**Table I. Comparison of Immunohistological Reactivity of Anti-OFA, Anti-placental VNR, and gp111a Antibodies**

Tissue	Anti-OFA $\alpha$ Chain		Rabbit/Rat <sup>‡</sup> Anti-OFA	Rabbit antiplacental VNR <sup>§</sup>	Anti-melanoma VNR mAbs LM142 and LM609	Integrin $\beta$ gp111a mAb VI-PL2
	mAb23C6	mAb13C2				
Human osteoclasts	+++	+++	++	++	+++	+++
Human osteoblasts	-	-	-	-	-	-
Human bone marrow	-	-	-	-	-	- (Platelets +)
Human kidney						
Glomerulus	±	±	±	±	±	+
Tubules	+	+	±	±	+	-
Vero monkey kidney cell line	++	++	nt <sup>¶</sup>	nt	++	-
Placenta	+	++	nt	nt	++	-/±
Cultured HUVE	++	++	nt	nt	++	++
Vascular endothelium	±	±	nt	nt	nt	+
Osteosarcoma cell lines <sup>  </sup>	-/±	-/±	nt	nt	-/±	-
Animal osteoclasts						
Rabbit	+++	+++	-	-	+++	-
Chicken	+++	-	-	±	nt	-

Immunohistological reactivity is scored from +++ (strongly positive) to - (negative) by comparison with appropriate positive and negative control antibodies for each tissue.

nt, not tested.

HUVE, Human umbilical vein endothelium.

\* Other tissues, tested in human and rabbit (Horton and Chambers, 1986) with 23C6 and 13C2, are all negative by immunohistology.

‡ Tested against formalin-fixed, wax-embedded tissues. Reactivity predominantly against  $\alpha$  chain by Western blotting (See Fig. 6).

§ Suzuki et al. (1986).

|| Low levels seen by immunofluorescence confirmed by immunoprecipitation (data not shown).

¶ Reactive by Western blot analysis; see Fig. 6.

VNR, as determined by immunohistological techniques (Figs. 1 and 8, Table I) using anti-OFA mAbs 13C2 and 23C6 (Horton et al., 1985b), LM142 and LM609 mAbs to melanoma VNR (Cheresh and Harper, 1987; Cheresh and Spiro, 1987) and heterosera to OFA and placental VNR (Suzuki et al., 1986) is identical for the two molecules.

Two further lines of evidence support our contention that OFA and VNR are closely related, or identical, molecules. First, epitope analysis (Nesbitt, S., and M. Horton, manuscript in preparation) has shown that anti-OFA monoclonal 23C6 and antimelanoma VNR antibody LM609 are directed against an identical antigenic site on both OC and melanoma receptors. Second, a rabbit polyclonal antiserum made against a synthetic peptide corresponding to the COOH-terminal 9 aminoacids, C-ENGEENSET, of the published cDNA sequence of the VNR  $\alpha$  chain (Suzuki et al., 1987) shows an identical immunological reactivity profile to antibodies 13C2 and 23C6; further, the antiserum immunoprecipitates both purified placental VNR (Pytela et al., 1987) and Vero OFA and an identically sized dimer from osteoclastoma tumor (Horton, M., and S. Nesbitt, manuscript in preparation).

The data presented in this paper are, thus, compatible with OFA being identical, or at least highly related, to the previously described integrin receptor, VNR (Pytela et al., 1985b; Suzuki et al., 1987; Fitzgerald et al., 1987b). The VNR is a member of a gene family that has been divided into three subsets according to the type of the shared  $\beta$  chain used (Hynes, 1987). One chain ( $\beta$ 1) defines the fibronectin receptor and the very late antigens, VLA1 to 6, of lymphocytes and some other tissues (Hemler, 1988; Takada et al., 1987; Sonnenberg et al., 1987).  $\beta$ 2 is restricted to leucocytes as the invariant chain (or CD18 antigen; see Hogg and Horton, 1987,

for relevant CD nomenclature) of the LFA-1 (CD11a), Mac-1 (CD11b) and gp150/95 (CD11c) antigens (Springer et al., 1987). The third group has platelet gp111a as its  $\beta$ ( $\beta$ 3) chain (Hynes, 1987; Phillips et al., 1988; Ginsberg et al., 1988) and includes the VNR and the gp11b/11a complex, the latter being largely restricted to platelets. As with the other integrin  $\alpha$  and  $\beta$  chains, OFA/VNR  $\alpha$  and  $\beta$  show a moderate degree of NH<sub>2</sub>-terminal amino acid homology with the other members of the gene family (see Fig. 7, legend and Results).

This study has highlighted some points relating to the biochemistry of VNR. First, two low molecular mass chains ( $\alpha'$ ), derived from the higher molecular mass component of the heterodimer (the  $\alpha$  chain) are frequently observed in our studies using immunoprecipitation with 13C2/23C6 mAbs from Vero cells. This contrasts with previous reports on the structure of VNR where only one  $\alpha'$  chain has been observed (Pytela et al., 1985b; Cheresh and Harper, 1987), but is similar to the finding of Sonnenberg et al. (1987) on the analogous platelet gpIc'-11a complex (now termed VLA-6) where two gpIc'-derived light chains are observed. Variation in glycosylation pattern (J. Davies, unpublished observations) does not seem to account for these differences as tunicamycin or endoglycosidase treatment fails to eliminate the disparity in molecular size. It is possible that it represents a species or tissue difference, the majority of our biochemical studies being carried out using a monkey kidney cell line, with differences in protein sequence of the Vero-derived OFA/VNR  $\alpha$  chain possibly occurring outside the region sequenced in this paper, and could account for the observations. A further possibility is that the production of light chains of two sizes could result from the use of minor extra proteolytic cleavage sites in the  $\alpha$  chain as have been reported for gp11b (Loftus et al., 1988), differential  $\alpha$  chain mRNA splicing (Suzuki et



al., 1987) or the use of more than one  $\alpha$  chain of a very similar size and structure in the VNR/OFA heterodimer. The rapidly increasing complexity of the integrin superfamily makes the existence of further VNR-like chains a real possibility.

The question arises as to the role of an integrin receptor (OFA/VNR) in bone? Two key observations provide clues as to its possible function. First, antibodies to the receptor can inhibit bone resorption and osteoclastic motility (Chambers et al., 1986) in a manner similar to the hormone calcitonin (Chambers and Magnus, 1982); this strongly suggests the involvement of an OFA-ligand interaction in the regulation of bone resorption. Second, the tissue distribution of OFA in bone is restricted to mature OCs (Horton et al., 1985b; Horton and Chambers, 1986) and their immediate precursors during embryonic development and adult life (Simpson and Horton, 1989). This points to a function involving recognition of a developmentally regulated protein ligand of specific segregation to bone; this might influence OC precursor homing, differentiation, fusion, and/or OC cellular adhesion to organic matrix of bone (and, *vide infra*, cell function in bone resorption). Vitronectin is a serum protein and not a prominent component bone, at least at an immunological level (Horton, M., unpublished observations; Hayman et al., 1983). Data from Cheresch and Spiro (1987) showed that VNR had a wider ligand binding specificity than originally reported (Pytela et al., 1985b); this suggests that OFA/VNR in bone could recognize an alternative protein to vitronectin. A candidate ligand for VNR with a tissue distribution limited to bone is osteopontin (Oldberg et al., 1986). It (a) contains the consensus Arg-Gly-Asp recognition sequence (Pierschbacher and Ruoslahti, 1984) common to the ligands of the majority of integrin receptors (Ruoslahti and Pierschbacher, 1987) including VNR (and OFA; this paper); (b) is synthesized by osteoblasts (Mark et al., 1987); (c) regulated by calcitropic factors (Prince and Butler, 1987; Noda et al., 1988); (d) is found by *in situ* mRNA hybridization in bone anlagen just before OC invasion (Nomura et al., 1988) and is expressed at the two other sites, placenta and kidney (Nomura et al., 1988) where high density OFA/VNR is localized (this paper; Horton et al., 1985b); and (e) can mediate cell adhesion (of fibroblasts) (Somerman et al., 1987).

Preliminary analysis of the cellular proteins capable of binding osteopontin-derived synthetic RGD-containing peptides (Davies, J., unpublished observations) demonstrates that a heterodimer with the characteristics of OFA/VNR is capable of acting as a receptor for osteopontin. Whether osteopontin is the natural ligand for OFA/VNR *in vivo* and what the exact role of such an interaction might be is currently under investigation. For example, can RGD-containing peptides and bone proteins influence OC differentiation and resorptive activity *in vitro*?

In this paper we report on the structure of a cell surface molecule, OFA, which is expressed at high levels by OCs (and some other tissues) and exhibits extensive homology/identity to the previously described integrin receptor VNR (Pytela et al., 1985b; Suzuki et al., 1987; Fitzgerald et al., 1987b). Previous functional studies using anti-OFA monoclonal antibodies (Chambers et al., 1986) point to a role for OFA in the regulation of bone resorption. The importance of OFA/VNR, and possibly other integrins and their ligands, in normal bone development and function, or indeed in pathogenesis of bone disease, remains to be fully evaluated.

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