Effect of VCAM-1 on the differentiation into osteoclast

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SYNOPSIS

In Orthodontic therapy, osteoclast precursors such as monocytes and macrophages migrate from vessels into the periodontium and then differentiate into osteoclasts, causing bone resorption. Focal adhesion kinase (FAK) is a 125-kDa non-receptor type tyrosine kinase that localizes to focal adhesions. FAK is involved in osteoclastic bone resorption. Vascular cell adhesion molecule-1 (VCAM -1) is bound to VLA-4(very late antigen-4) which is kind of the α_4 β1-integrin, and recruits monocytic osteoclast progenitors and elevates local osteoclast activity.

In our present study we focused on VCAM-1 /α_4 integrin-mediated the differentiation into osteoclasts and found that this type of the differentiation was mediated through FAK. RAW267.4 expressed both α_4 integrins, and it was reported that expression of α_4 integrin and its counterreceptor, VCAM-1, was not enhanced in response to receptor activator of NF-B ligand (RANKL). Neutralizing antibodies against integrin α4 effectively inhibit the differentiation into osteoclasts and phosphorylation of FAK. These findings establish VCAM-1 regulate the differentiation into osteoclasts in bone.

Key words: *osteoclast, FAK, VCAM-1, RANKL*

INTRODUCTION

Tooth movement in orthodontic therapy is the result in the two events comprising bone remodeling; bone formation by osteoblasts and bone resorption by osteoclasts. Both processes are synchronized by inter- and intracellular signaling involving hormones, growth factors, and attachment receptors binding to the extracellular matrix (ECM) [1]. ECM is gradually increased in the pressure side and reduced in the tension side in orthodontic therapy [2].

Integrins are transmembrane glycoproteins consisting of α and β chains, and there are eight subfamilies based on β-chain structure, β1 to β8. VLA-4 (very late antigen: α4β1) and VLA-5 (very late antigen: α5β1) play the most central role in the adhesion between T cells and vascular endothelial cells [3,4]. The extracellular matrix protein, VCAM-1 is a member of the transmembrane immunoglobulin superfamily [5]. Proteolytic shedding of VCAM-1 also generates a soluble form of VCAM-1 [6]. The predominant receptor for VCAM-1 is integrin α4β1, which is expressed by many cell types of the hematopoietic lineage, including B and T lymphocytes, monocytes, eosinophils, and osteoblasts [7,8]. VCAM-1 is expressed by cytokine-activated osteoblasts [7] and VCAM-1-α4β1 binding plays an important role in mediating leukocyte adhesion and

transendothelial migration during inflammation [9,10], which may be the underlying mechanism for VCAM-1 function in differentiation [8].

The focal adhesion kinase (FAK) family kinases are major tyrosine kinases activated by β1-integrin engagement. FAK is involved in osteoclastic bone resorption and that tyrosine phosphorylation of p125FAK is critical for regulating osteoclast function [11].

The evidence implicating ECM in bone remodeling suggests that ECM is directly modulating differentiation and activity in osteoclast. Here we hypothesize that VCAM-1 have stimulatory effect on the differentiation into osteoclast. Our data identify VCAM-1 as a novel prompter of the differentiation into osteoclast. VCAM-1 enhanced RANKL stimulated differentiation into osteoclast and phosphorylation of FAK inRAW264.7 cells. α4-blocking antibodies effectively inhibit differentiation into osteoclast and phosphorylation of FAK enhanced by VCAM-1 in RAW264.7 cells.

We discovered a role of VCAM-1 in promoting the differentiation in bone.

MATERIALS AND METHODS

1. Cell culture

The RAW264.7 cell line as osteoclast precursors was obtained from Riken Cell Bank (Ibaragi, Japan) and maintained in the growth medium (RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Equitech-Bio Inc., TX, USA), 100 unit/ml penicillin G sodium, 100 μg/ml streptomycin and 292 mg/ml L-glutamine (Invitrogen Co., CA, USA)) at 37°C under 5% CO_2. During subculture, the cells were detached by Trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA-4Na) (Invitrogen Co., CA, USA).

2. Antibodies and reagents

Anti-mouse CD49d(9C-10 & R1-2) and Rat IgG2a, κ Isotype Ctrl were obtained from BioLegend (CA, USA) and recombinant mouse VCAM-1 obtained from R&D Systems (MN, USA). We used anti-FAK [pY576] phosphospecific antibody (Invitrogen Co., CA, USA), anti-FAK for a primary antibody of Western Blotting. Horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibodys were from Amersham Biosciences Co. (NJ, USA). The ECL detection kit was purchased from Millipore (MA, USA).

Recombinant human RANKL were purchased from Wako Pure Chemicals

(Osaka, Japan). Tartrate-resistant acid phosphatase (TRAP) buffer solution was prepared by mixing 0.1 M sodium acetate (Wako Pure Chemicals) and 50 mM tartaric acid (Wako Pure Chemicals) in a 1:1 ratio, followed by adjustment to pH 5. TRAP staining solution was prepared by mixing 50 ml of TRAP buffer solution, 5 mg of naphthol AS-MX phosphate (Sigma-Aldrich, MO, USA), 0.5 ml of N,N-dimethylformamide (Wako Pure Chemicals) and 25 mg of Fast Red Violet LB salt (Sigma-Aldrich).

3. TRAP staining

RAW264.7 cells were seeded on 96-well plate that immobilized VCAM-1 with 1, 10μg/ml, at a density of 3.0×10^3 cells/well and cultured for up to 3days in growth medium supplemented 50 ng/ml RANKL with or without neutralizing anti-mouse CD49d(9-C10 & R1-2) at 10μg/ml. Osteoclast formation was assessed by tartrate-resistant acid phosphatase (TRAP) staining. The cells were fixed in 10% paraformaldehyde / PBS (-) for 1 minutes and an acetone and ethanol mixture (1:1) for 30 seconds. The fixed cells were treated with the TRAP staining solution for 10 minutes, washed by water, and observed under a light microscope. TRAP positive multinucleated cells containing at least three nuclei were counted as osteoclast. Cell counts indicate the average of 4 wells.

4. Western blotting

5.0×10^5 cells were seeded on 24-well plates that immobilized VCAM-1 with 1μg/ml and incubated overnight in RPMI supplemented with 2% FBS and 10μg/ml neutralizing anti-mouse CD49d. The cells were stimulated by 50 ng/ml RANKL for the indicated times. The cells were directly lysed by SDS-sample buffer and the lysate were sonicated with a Branson digital sonifier 450 (Branson Ultrasonics, Danbury, CT, USA). The samples were boiled at 99°C for 3 minutes and the same amount of samples were loaded and separated by 8% SDS-PAGE. After Western transfer of the proteins, PVDF Membrane (Immbilon-P, Millipore) was blocked with 5% BSA / TBS-T. The membrane was incubated overnight with primary antibody in 5% BSA / TBS-T, washed with TBS-T, incubated for 1 hour with HRP-labeled secondary antibody in 1% BSA / TBS-T, and finally washed with TBS-T. Membranes were developed by Immobilon Western Chemiluminescent HRP Substrate (Millipore). The same membranes were stripped and reprobed with antibody recognizing unphosphorylated form of the protein to normalize the results.

RESULTS

1. Effect of RANKL on integrin expression in RAW264.7 cells

First we tested the effects of RANKL stimulation on integrin expression. β1- and α4- integrin expressed in murine preosteoclast cell line (RAW264.7 cells) using flow cytometry. Interestingly, expression of β1- and α4- integrins did not change after RANKL stimulation. Therefore, RAW264.7 cells, given physical proximity, could interact directly via VCAM-1-α4β1 binding (Figure 1).

2. VCAM-1 enhanced the differentiation into osteoclasts in RAW264.7 cells

Several ECM and cytokines contribute to the process of osteoclastogenesis. We analyzed VCAM-1 function in osteoclastogenesis by pre-coating culture plates with recombinant VCAM-1 before seeding RAW264.7 cells. 1, 10μg/ml VCAM-1 coating increased the number of TRAP positive osteoclasts compared to BSA coating in RANKL stimulated RAW264.7 cells (Figure 2 A and B).

3. Effect of VCAM-1 on the phosphorylation of FAK in RAW264.7 cells

It has been reported that FAK plays a key role in regulating osteoclastic and bone resorption [11]. When RAW264.7cells were cultured in the presence of 100 ng/ml RANKL, FAK was phosphorylated in a time-dependent manner, with maximum phosphorylation observed after 30 min (Figure. 3). The total amount of

the FAK protein was not affected under any of the experimental conditions used (Figure. 3).

To determine if FAK may be involved in VCAM-1-mediated co-stimulation with RANKL in RAW264.7 cells, we examined the induction of FAK tyrosine phosphorylation upon stimulation with RANKL alone, or RANKL in the presence of VCAM-1. VCAM-1 coating enhanced tyrosine phosphorylation compared to RANKL alone (Figure 4).

4. α4-integrin regulate the osteoclasts differentiation

We examined roles of α4-integrin, a ligand of VCAM-1 were analyzed by monitoring the effects of anti- α4-integrin mAb on the differentiation into osteoclasts in RAW264.7 cells. In addition the inhibitory α4-integrin-specific mAb, but not control IgG mAb on VCMA-1 potently blocked the differentiation into osteoclasts induced by RANKL while no change on BSA was detected (Figure 5 A and B). Finally we tested whether the FAK pathway was also involved in VCAM-1 stimulation of the differentiation into osteoclasts inRAW267.4 cells. The inhibitory α4-integrin-specific mAb also deceased phosphorylation of FAK compared to VCAM-1-mediated co-stimulated with RANKL (Figure 6).

DISCUSSION

A primary concern of orthodontists has been the cellular or sub-cellular changes that occur in the periodontal ligament and alveolar bone. Thus, dramatic changes in the level of ECM and proteases in gingival tissue contour should always follow tooth movement [12-14]. ECM is gradually increased in gingival tissue in orthodontic therapy [2] and their effects on these conditions implicate them as osteoclastogenic promoters. However, their effect on osteoclastogenesis has not been thoroughly investigated. To delineate the role of matrix proteins in osteoclast formation, we studied the effects of physically adsorbed VICAM-1 on the differentiation into osteoclasts in RAW267.4 cells. Although α4- and β1-(data not shown) integrins expressed, RANKL stimulation did not enhance the expression of β1- and α4- integrins in RAW264.7 cells (data not shown). There was not the expression of VCAM-1 after RANKL-stimulation in RAW264.7 Integrins play major roles in intercellular adhesion and adhesion of cells to extracellular matrix proteins [15,16]. VCAM-1 is not sufficient for osteoclast differentiation without RANKL-stimulation in RAW264.7 cells. VCAM-1enhanced the number of TRAP positive osteoclasts cells compared to BSA. α4 - integrin adhere to VCAM-1 dependent on bivalent metal cations. This

adhesion is regulated by intracellular signals. These finding led us to hypothesize that the effect of VCAM-1 on osteoclastogenesis depend on outside-in signaling pathway such as activation of FAK. FAK activity elicits intracellular signal transduction pathways that promote the turn-over of cell contacts with the extracellular matrix, promoting cell migration and osteoclasts differentiation [11]. Therefore, we examined the co-stimulatory effect of α4integrin-mediated FAK tyrosine phosphorylation. RANKL co-stimulated with α4integrin enhanced the phosphorylation of FAK at y576 in RAW2964.7 cells. The inhibitory α4-integrin specific mAb, but not control IgG antibody, potently blocked t the phosphorylation of FAK at y576 on RAW267.4 cells. These data suggest that outside-in signal of VCAM-1/α4-integin enhanced activation of FAK.

Interesting findings of our study were co-stimulatory effect of α4- integrin on RANKL induced osteoclast differentiation on VCAM-1. When RAW264.7 cells were cultured with anti-α4 specific antibody, the decease in osteoclast number was on VCAM-1, but not on BSA. FN (fibronectin) has most pronounced inhibitory effect on osteoclastogenesis in RAW267.4 cells [17]. αvβ3-integrin enhances cell adhesion to FN and block osteoclastogenesis [17]. FN/αvβ3 inhibits osteoclasts formation by preventing pre-osteoclasts fusion. Our data

suggest that VCAM-1/α4 signaling is co-stimulatory factor in RANKL induced osteoclasts differentiation.

Taken together, these results highlight the important contribution of matrix proteins in modulating bone remodeling. An important outcome of this study is the co-stimulatory effect of VCAM-1 on osteoclasts formation. Increased expression of VCAM-1 in orthodontic treatment results in promoting bone remodeling and moving teeth.

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Figure legends

Fig. 1 - Effect of RANKL on integrins expression in RAW264.7 cells. RAW264.7 cells were seeded in a 6-well plate (2.0×10^5 cells/well), incubated in RPMI (10% FBS) with RANKL stimulation for 24 h. RAW264.7 cells were stained with antibodies against β1-, α4-integrins and VCAM-1.

Fig 2 - RANKL-induced RAW264.7 cell into osteoclasts differentiation on precoated plate by VCAM-1. VCAM-1 was coated 96-well plate (10 μg/ml with 100 μl/well) for 1 hour incubation at room temperature. RAW264.7 cells (3.0×10^3 cells/well) were cultured in a plate and stimulated with 50 ng/ml RANKL for 3 days. Cells were then subjected to TRAP assays.

(A) TRAP staining was performed.

(B) The number of TRAP-positive multinucleated cells contain three or more nuclei were scored. Cell counts indicate the average of the date for 4 wells (***, $p < 0.01$).

Fig. 3 - Phosphorylation of FAK in RAW264.7 cells. RAW264.7 cells (5.0×10^5 cells/well) were cultured in a 24-well plate for 24hours, treated with 2%

FBS and then treated with 50 ng/ml RANKL for 0, 5, 10, 15, 30, 60 minutes. Cell lysates were separated on SDS-PAGE and blotted with anti-phospho FAK (y576) antibody. They were then visualized with Immobilon Western Chemiluminescent HRP Substrate. The same membranes were stripped and reprobed with anti-FAK antibody.

Fig. 4 - Tyrosine phosphorylation of FAK by RANKL co-stimulation with VCAM-1 in RAW264.7 cells. VCAM-1 was coated 24-well plate (1 μg/ml with 500 μl/well) for 1 hour incubation at room temperature. RAW264.7 cells (5.0×10^5 cells/well) were cultured in a plate for 24 hours, treated with 2% FBS and stimulated with 50 ng/ml RANKL for 30 minutes. Cell lysates were separated on SDS-PAGE and blotted with anti-phospho FAK (y576) antibody. They were then visualized with Immobilon Western Chemiluminescent HRP Substrate. The same membranes were stripped and reprobed with anti-FAK antibody.

Fig. 5 - Effect of Anti-mouse CD49d/CD29 on VCAM-1 and RANKL-induced the differentiation into osteoclasts. VCAM-1 was coated 96-well plate (10 μg/ml with 100 μl/well) for 1 hour incubation at room temperature. RAW264.7

cells (3.0×10^3 cells/well) were cultured in a plate, treated with anti-CD49d (10μg/ml) and stimulated with 50 ng/ml RANKL for 3 days. Cells were then subjected to TRAP assays.

(A) TRAP staining was performed.

(B) The number of TRAP-positive multinucleated cells contain three or more nuclei were scored. Cell counts indicate the average of the date for 5 wells (***, $p < 0.001$).

Fig. 6 - Effect of anti-mouse CD49d in tyrosine phosphorylation of FAK immobilizing VCAM-1. VCAM-1 was coated 24-well plate (1 μg/ml with 500 μl/well) for 1 hour incubation at room temperature. RAW264.7 cells (5.0×10^5 cells/well) were cultured in a plate, treated with anti-CD49d (10μg/ml) and stimulated with 50 ng/ml RANKL for 30 minutes. Cell lysates were separated on SDS-PAGE and blotted with anti-phospho FAK (y576) antibody. They were then visualized with Immobilon Western Chemiluminescent HRP Substrate. The same membranes were stripped and reprobed with anti-FAK antibody.

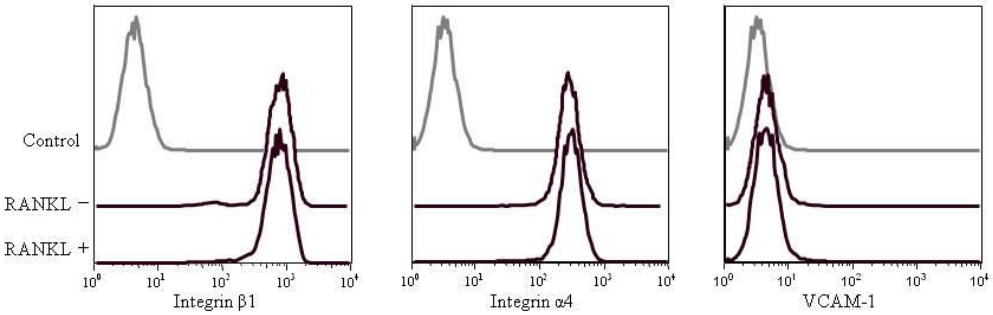


Fig. 1 Effect of RANKL on integrins expression in RAW264.7 cells.

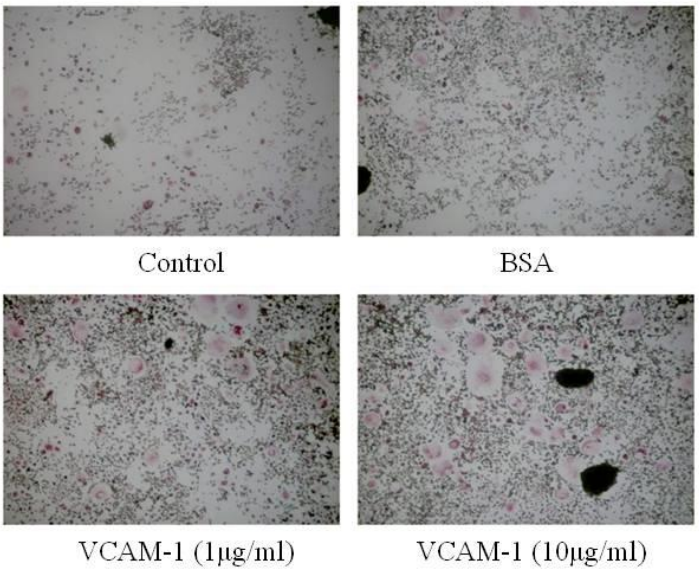


Fig 2 RANKL-induced RAW264.7 cell into osteoclasts differentiation on precoated plate by VCAM-1. (A) TRAP staining was performed.

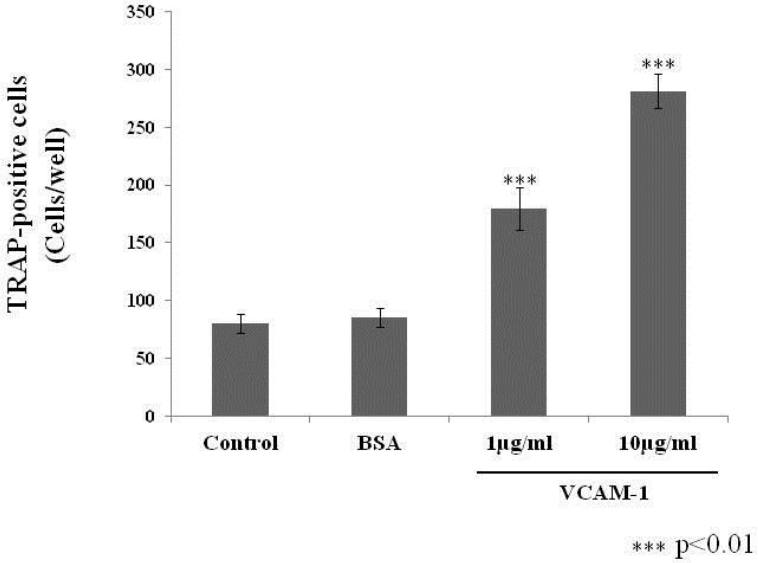


Fig 2(B) The number of TRAP-positive multinucleated cells contain three or more nuclei were scored. Cell counts indicate the average of the date for 4 wells (***, $p < 0.01$).

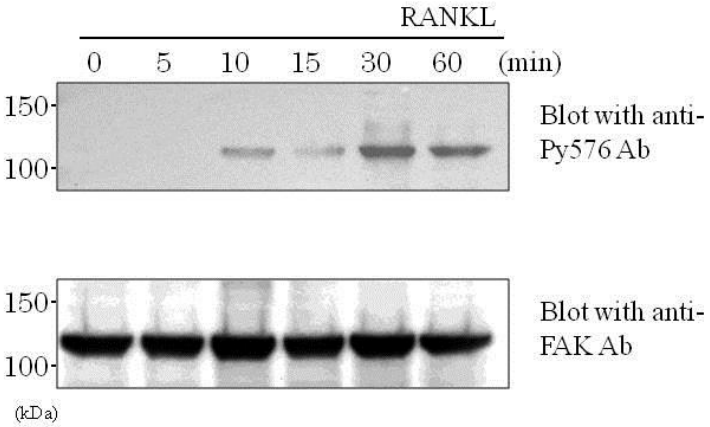


Fig. 3 Phosphorylation of FAK in RAW264.7 cells.

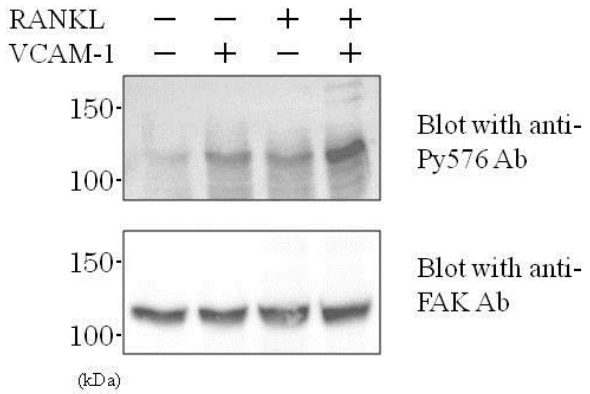


Fig. 4 Tyrosine phosphorylation of FAK by RANKL co-stimulation with VCAM-1 in RAW264.7 cells.

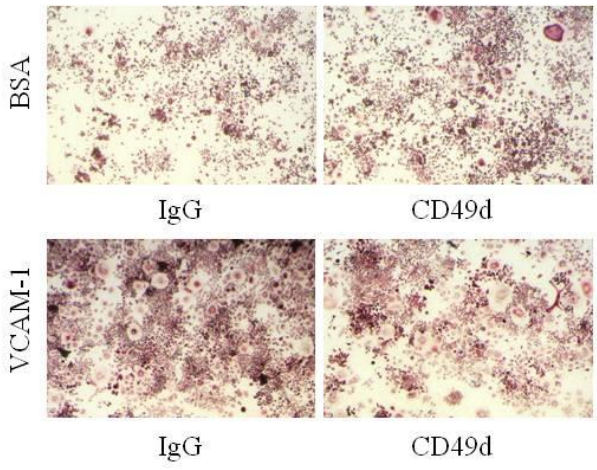
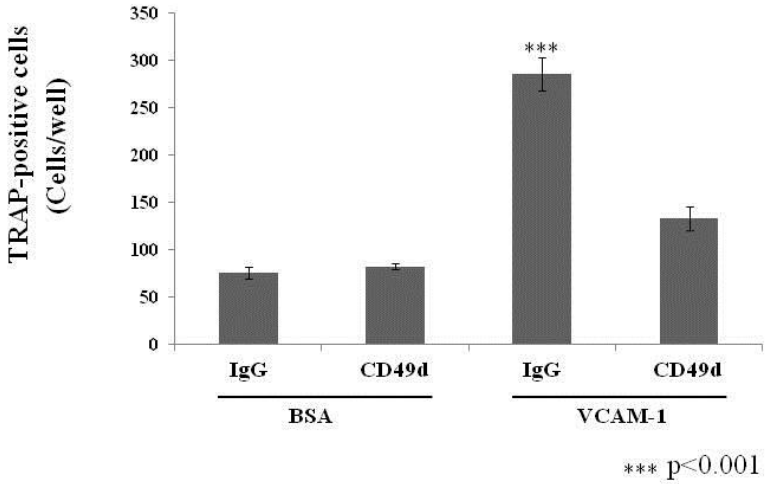


Fig. 5 (A) Effect of Anti-mouse CD49d/CD29 on VCAM-1 and RANKL-induced the differentiation into osteoclasts. (A) TRAP staining was performed.



(B) The number of TRAP-positive multinucleated cells contain three or more nuclei were scored. Cell counts indicate the average of the date for 5 wells (***, $p < 0.001$).

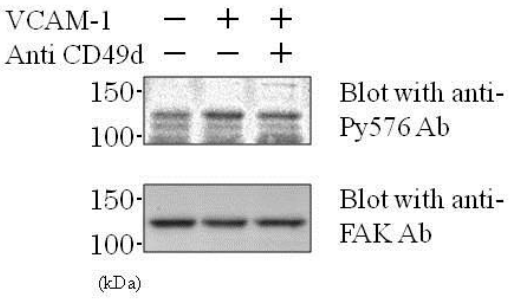


Fig. 6 Effect of anti-mouse CD49d in tyrosine phosphorylation of FAK immobilizing VCAM-1.