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$Alpha_VBeta_5$ integrins mediates *Pseudomonas fluorescens* interaction with A549 cells

Elisabetta Buommino[1], Marina Di Domenico[2], Iole Paoletti[1], Alessandra Fusco[1], Vincenza De Gregorio[1],Valentina Cozza[3], Antonietta Rizzo[1], Maria Antonietta Tufano[1], Giovanna Donnarumma[1]

[1]Department of Experimental Medicine, Section of Microbiology and Clinical Microbiology, Second University of Naples, Italy, [2]Department of General Pathology, Faculty of Medicine of Naples, Second University of Naples, Naples, Italy, [3]Department of business management, University of Naples Parthenope, Naples, Italy

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1. ABSTRACT

Interaction of pathogenic bacteria with human cells is usually an essential step in the infection process. The bacterial invasion is stimulated by microbial binding to mammalian extracellular matrix proteins such as vitronectin, fibronectin or integrins. We have recently shown that some strains isolated from a clinical environment are able to grow at/or above 37°C. In particular, we demonstrated that *P. fluorescens* AF181 binds specifically to the surface of A549 human respiratory epithelial cells and that adhesiveness modulates the inflammatory response. In this study, the involvement of $Alpha_VBeta_5$ integrins and its respective natural ligand vitronectin (VN) in *P. fluorescens* AF181 adherence and invasion was examined. The host cell cytoskeleton and cellular tyrosine kinases seem to be solicited during the *P. fluorescens*-respiratory cell interaction; consequently, cytochalasin D and genistein decreased the bacterial adherence and internalization. Gene silencing of αv, β5 integrins and vitronectin reduced *P. fluorescens* adherence and internalization to A549 cells. Taken together, these findings suggest that $Alpha_VBeta_5$ integrins and their natural ligand VN are involved in *P. fluorescens* adherence and invasion in human epithelial cells.

2. INTRODUCTION

Interaction of pathogenic bacteria with human cells is usually an essential step in the infection process. This interaction can comprise adherence, injection of virulence factors through the type III secretion system, transduction of signals to the target cell through the signaling machinery of the host cells, and induction of internalization (1). Intracellular invasion by several bacterial pathogens is stimulated by microbial binding to mammalian extracellular matrix (ECM) proteins such as vitronectin (VN) or fibronectin (FN) and to integrins, their natural cellular receptors (2,3). Integrins are a class of heterodimeric transmembrane receptors, consisting of two glycoprotein sub-units, α and β, involved in ECM-cell interactions (4). Following ligand recognition, integrins undergo clustering and conformational changes that result in recruitment of a number of intracellular signaling molecules. This is followed by activation of several signaling cascades and, consequently, regulates vital cellular functions, such as proliferation, differentiation, migration, cytokine release, etc. (5). Different ab integrin heterodimers are expressed in normal lung epithelial cells. These receptors recognize an array of ECM proteins: collagen I, tenascin C, laminins 5, 10, 11, osteopontin,

fibronectin, vitronectin, etc. (6) It has been established that lung integrins are critical for tissue development, maintaining epithelial integrity, repair of damaged tissue, and regulation of inflammatory responses and tissue remodelling. Previous studies have demonstrated that the expression of integrin receptors in the pulmonary epithelium can change under various pathological conditions, such as injury, inflammation, or malignant transformation.(7,8,9) Some pathogenic micro-organisms (e.g. *Streptococcus pyogenes* and *Pneumocystis carinii)* are able to increase integrin expression in infected respiratory epithelial cells, and the resulting events have been implicated in microbial pathogenesis. (10,11,12) However, specific mechanisms underlying the effects of pathogens on integrin expression as well as the functional consequences of integrin alterations for the pulmonary epithelium are poorly understood.

Pseudomonas aeruginosa is an opportunistic pathogen which cause severe pulmonary infections in immunocompromized patients. It is the leading cause of ventilator-associated pneumonia in intensive care units with high mortality rates. (13) P. aeruginosa is the major cause of chronic pulmonary infection in cystic fibrosis patients that determines the overall prognosis of this genetic disease, (14) as well as a significant cause of exacerbations of chronic obstructive pulmonary disease (COPD) (15). Conversely, little attention has been given to other species of the same genus, like Pseudomonas fluorescens. *P. fluorescens* is an uncommon cause of human infection. During 2000–2004, only 35 cases of *P. fluorescens* bloodstream infection were reported to the National Nosocomial Infections Surveillance System, a voluntary reporting system of 300 hospitals across the United States. Although considered to be of low virulence, *P. fluorescens* has been reported to cause blood transfusion–associated septicemia (at times, fatal), and catheter-related bacteremia among patients with cancer (16). In addition, its incidence in nosocomial infections has been considerably increased during the last few years. (17)

P. fluorescens has long been considered as a psychrotrophic microorganism, unable to grow at temperatures over 32°C. However, we have recently shown that some strains isolated from a clinical environment are able to grow at or above 37°C. (17) In particular, we demonstrated that *P. fluorescens* AF181 binds specifically to the surface of cultured A549 human respiratory epithelial cells and that adhesiveness modulates the inflammatory response. (18)

In this study, involvement of αvβ5 integrins and its respective natural ligand vitronectin (VN), in *P. fluorescens* AF181 adherence and invasion was examined.

3. MATERIAL AND METHODS

3.1. Bacterial strain

Pseudomonas. fluorescens AF181 was grown in Luria-Bertani (LB) medium (Becton, Dickinson, Co; Milano, Italy) at 32°C, as previously described (18). Bacteria were grown on a rotary shaker (180r/min). In some experiments, bacteria were harvested by centrifugation in the early stationary phase and re-suspended in LB medium at an OD600 of 0.5 (BioPhotometer plus Eppendorf; Milano, Italy).

3.2. Cell culture

The human A549 lung epithelial cell type II line (ATCC-CCL185TM) (American Type Culture Collection Manassas, VA) was grown in F12K medium (Invitrogen, Carlasbad, CA) supplemented with 10% (v/v) fetal calf serum (Invitrogen Carlasbad, CA) and 100 UI/ml of penicillin and 100μg/ml of streptomycin (Invitrogen, Carlasbad, CA). Routinuosly, cells were seeded in 6-well tissue culture plates and used at 80% confluence.

3.3. Adhesiveness assay

For adhesiveness assays, semiconfluent monolayers (approximately 8x105 cells) were grown overnight with antibiotics, washed with fresh medium without antibiotics and infected with exponentially-growing *P. fluorescens* strain AF181 at a multiplicity of infection (MOI) of 100 bacteria/cell. Infected monolayers were centrifuged at 600×g for 5 min and incubated at 37°C in 5% CO2 for 3h to favor adhesion. At the end of the experiment, infected monolayers were washed three times with F12K medium to remove non-adherent bacteria. Total number of cell-associated bacteria was determined by lysing the monolayers with 0.1% Triton X-100. Solubilized bacteria were counted (CFUs) by spreading serial dilutions on LB agar plates and incubating at 37°C overnight. Adhesion efficiency was calculated as the ratio of the number of cell-associated bacteria and the number of bacteria used to infect cell monolayers.

3.4. Bacterial internalization assays

As described above, semiconfluent A549 cell cultures were infected with *P. fluorescens* AF181 (MOI 100). After 2 h of incubation at 37°C infected monolayers were extensively washed with sterile PBS and further incubated for other 2 h in F12K medium supplemented with gentamicin sulphate (250 μg ml-1) (Sigma-Aldrich; Milano, Italy) in order to kill extracellular bacteria. We had previously determined that at this concentration gentamicin inhibits 99.9% of *P. fluorescens* AF181 growth. At the end of the experiments, infected monolayers were extensively washed in PBS, then lysed with a solution of 0.1% Triton X-100 (Sigma-Aldrich; Milano, Italy) in PBS for 10 minutes at room temperature to count internalized bacteria. Aliquots of cell lysates were serially diluted and plated on LB agar to quantify viable intracellular bacteria (cfu ml-1). The efficiency was calculated as the ratio of the number of cell-internalized bacteria and the number of bacteria used to infect the cell monolayers. Evaluation of gentamicin toxicity towards A549 cells was assessed by an XTT-based colorimetric assay (Cell Proliferation Kit; Roche, Milan, Italy). In the experiments in which genistein (Sigma-Aldrich; Milano, Italy) (10 μg/ml) (Protein tyrosine kinases inhibitor) or cytochalasin D from Zygosporium mansonii Sigma-Aldrich; Milano, Italy) (10 μg/ml) (actin polymerization inhibitor) were used, epithelial cells were pretreated with the inhibitors for 1 h before the addition of bacterial suspension. Bacteria interacting with or

Table 1. Primer sequences

Gene	Primers sequence	Conditions	Product size (bp)
alphav	5'- TAAAGGCAGATGGCAAAGGAG -3' 5'- CAGTGGAATGGAAACGATGAGC -3'	5''at 94°C, 10" at 64°C, 20''at 72°C for 40 cycles	510
beta5	5'- CAGCCCCGGCTACCTGGGCAC -3' 5'- CTGGCACAGGAGAAGTTGTCGCAC -3'	5''at 94°C, 10" at 60°C, 8''at 72°C for 40 cycles	200
vitronectin	5'- CGAGGAGAAAAACAATGCCAC -3' 5'- GAAGCCGTCAGAGATATTTCG -3'	5''at 94°C, 10" at 58°C, 21''at 72°C for 40 cycles	502
β-actin	5'- TGACGGGGTCACCCACACTGTGCCCATCTA -3' 5'- CTAGAAGCATTGCGGTGGACGATGGAGGG -3'		243

internalized by human cells in the presence of inhibitors were enumerated as described above. At the concentrations used, none of these components affected epithelial cell or bacterial viability.

3.5. Western blot analysis and Immunoprecipitation

Semiconfluent monolayers (approximately 8x105 cells) were grown overnight with antibiotics, washed with fresh medium without antibodies and infected with exponentially-growing *P. fluorescens* AF181 at a multiplicity of infection (MOI) of 100 bacteria/cell. Infected monolayers were centrifuged and incubated at 37°C in 5% CO2 for 2,4 and 6 h to favor adhesion. At the end of the experiment, infected monolayers were washed three times with F12K medium to remove non-adherent bacteria.

The cells were scraped with 1 ml of PBS and the cell pellet was homogenized with 300 ml of ice-cold buffer [137mM NaCl, 1% glycerol 10%, 20mMTris-HCl pH 8, EDTA 5mM, Triton X 100 1%, 1 mM NaP2O7, 10mM b-glycerophosphate (CH2OH)2CHOP(O)(NaO)2] supplemented with 2µg/ml of aprotinin, 20µg/ml leupeptina, 1mM phenylmethylsulfonyl fluoride (PMSF), 1mM sodium orthovanadate and 10mM NaF, 1mM EGTA. Total extracts were cleared by centrifugation for 30 min at 4° C at 10,000 g and assayed for protein content by Bradford's method. Fifty micrograms of protein from each cell lysate were separated by a 7 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes before staining the filters with 10% Ponceau S solution for 2 min to verify equal loading and transfer efficiency. Blots were blocked overnight with 5% non-fat dry milk and then incubated with anti-αv, β3, β5 and vitronectin monoclonal antibodies (Santa Crutz, Milan, Italy) , 1 mg/ml in Tris-buffered saline (TBS) (150mM NaCl and 20mM Tris–HCl, pH8) for 2 h at room temperature. After washing with 0.1% Tween-20 PBS, the filters were incubated with 1:2000 peroxidase-conjugated antimouse/ rabbit immunoglobulins for 1 h at 22° C. They were thoroughly washed and then analysed using the ECL system (Amersham, Milan, Italy). In the immunoprecipitation experiments, 400 µg of protein derived from cell lysates were immunoprecipitated with 5 µg/ml of anti-β3 and anti-β5 polyclonal antibodies overnight at 4 °C. The immunoprecipitated proteins were recovered by 30 µL of a 1:1 suspension of protein A-Sepharose for 2 h at 4 °C and analysed for the av content by Western blot as above described.

3.6. siRNA for αv, and β5 integrin subunits and vitronectin

Cells were grown to 60–70% confluence in 24 multiwell plates and transfected with 30 nmol specific siRNA constructs for αv and β5 integrin (24 h) and 50 nmol for vitronectin (48 h), according to the manufacturer's instructions (QIAGEN, Milan, Italy). Following silencing, cells were treated with bacterial suspension for adhesiveness and internalization assays as previously described.

3.7. Real-time PCR analysis

Following silencing, total RNA was isolated by using the High Pure RNA Isolation Kit (Roche; Milano, Italy). Two hundred nanograms of total cellular RNA were reverse-transcribed (Expand Reverse Transcriptase, Roche; Milano, Italy) into complementary DNA (cDNA) using random hexamer primers (Random hexamers, Roche; Milano, Italy), at 42°C for 45 min, according to the manufacturer's instructions. Real time PCR was carried out with the LC Fast Start DNA Master SYBR Green kit (LightCycler 2.0 Instrument, Roche; Milano, Italy) using 2 µl of cDNA, corresponding to 10 ng of total RNA in a 20 µl final volume, 3 mM $MgCl_2$ and 0.5 µM sense and antisense primer. A melting curve was made at the end of each amplification to ensure the absence of non-specific reaction products. The accuracy of mRNA quantification depends on the linearity and efficiency of the PCR amplification. Both parameters were assessed using standard curves generated by increasing amounts of cDNA. Quantification is based on the threshold cycle values, which are measured in the early stage of the exponential phase of the reaction, and by normalization to the internal standard curve obtained with the housekeeping β-actin gene to avoid discrepancies in input RNA or in reverse transcription efficiency. The PCR products were examined on 1.8% agarose gel.

3.8. Statistics

All the experiments were conducted three time with at least three replicate for group. A difference in mean values was deemed significant if the P values was <0.05 or highly significant if the P values was 0.01. The three experimental groups were compared using a one-way analysis of variance. Post hoc group comparison were conducted using the Student-Newman-Keels test.

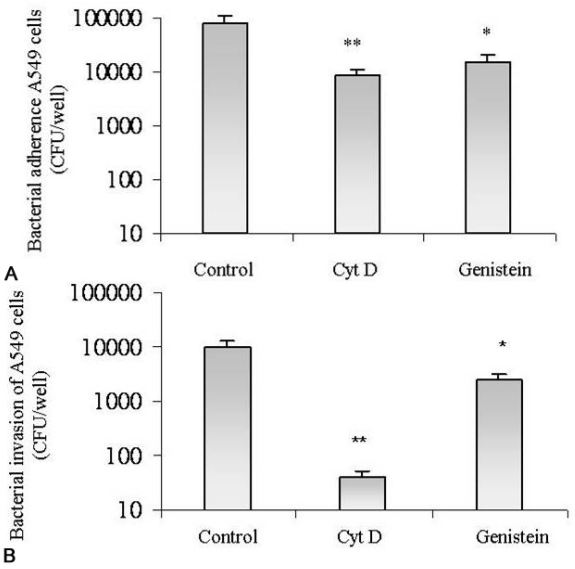

Figure 1. *P. fluorescens* adhesion and invasion assays. A549 cells infected with *P. fluorescens* were incubated with inhibitors for 3 h at 37 °C. Number of bacteria associated with A549 cells (panel A) and bacteria internalized (panel B) was determined by host cell lysis, plating, and counting CFU/well. Control: untreated cells. Cyt D: cytochalasin D; Genist: Genistein. The data shown are representative of three different experiments ± S.D. Error bars represent standard deviations. * $P < 0.05$, ** $P < 0.01$, vs. control.

4. RESULTS

4.1. *P. fluorescens* interaction with A549: adherence and invasion

Cellular tyrosine kinases and cytoskeletal actin microfilament arrangement have been previously shown to be involved in Pseudomonas internalization by mammalian cells (19,20,21). Here we tested bacterial infection in the presence of genistein, the tyrosine kinase inhibitor or cytochalasin D, the F-actin depolymerization factor. Treatment of A549 respiratory cells with genistein significantly affected bacterial adherence and internalization of *P. fluorescens* AF181 ($P < 0.05$). *P. fluorescens* AF181 adherence and invasion were reduced by 79.49 and 69.77%, respectively (Figure 1A and 1B). Treatment of A549 cells with cytochalasin D significantly decreased both bacterial adherence and internalization ($P < 0.01$), too. Namely, *P. fluorescens* AF181 adherence was reduced by 84.41 % (Figure 1A) and the specific internalization by 99.57 % (Figure 1B). It is important to note that at the concentrations used, none of these components affected A549 cell or bacterial viability (data not shown).

4.2. *P. fluorescens* up-regulates the αvβ5 integrin in A549 cells

To address the question whether *P. fluorescens* AF181 infection can modulate the expression of integrin receptors in lung epithelial cells the levels of α5, αv, β3, and β5 integrin subunits were evaluated. A549 cells were stimulated with *P. fluorescens* AF181 for 2, 4, or 6 h and the expression of αv, β3, and β5 integrin subunits was examined using real time PCR and western blot analysis. As shown in Figure 2A and 2B, the expression of αv, β3 and β5 integrin subunits was significantly up-regulated after 2 h of *P. fluorescens* AF181 infection. In contrast, no modulation of α5 was demonstrated (data not show). To identify the type of VnR involved in the *P. fluorescens*-dependent av up-regulation, uninfected or P- fluorescens AF181 infected cells lysates were analysed by immunoprecipitation using anti-β3 mAbs and anti-β5 polyclonal antibodies. As shown in Figure 3 a strong increase of av content was found in anti-β5 immunoprecipitate derived from *P. fluorescens* AF181 infected cell lysates, thus suggesting that *P. fluorescens* induces up-regulation of the αvβ5 integrin.

4.3. Effects of gene silencing of αv and β5 integrin subunits and the αvβ5 ligand VN, on *P. fluorescens* adherence and invasion

To determine the potential implication of αvβ5-VN systems in the interactions between *P. fluorescens* AF181 and human respiratory cells, the effect of integrin subunits and VN silencing on adherence and invasion assays was analysed. Real time-PCR and western blot analysis confirmed that the siRNA construct reduced integrin subunits and VN expression compared to untreated control levels. (Figure 4A and 4B)

P. fluorescens AF181 adherence to A549 cells and specific internalization were significantly decreased by 99.4 % and 83.8 %, respectively, after av integrin subunit silencing, and by 99 % and 70.5 %, respectively, after β5 integrin subunit silencing. Furthermore, gene silencing of VN strongly inhibited both *P. fluorescens* AF181 adherence and invasion (99.4 %, 81.3 %) to A549 cells (Figure 5A and 5B).

5. DISCUSSION

We have recently presented evidence that *P. fluorescens* binds specifically to the surface of cultured A549 human respiratory epithelial cells and that adhesiveness modulates the inflammatory response (18). In this study, we investigated *P. fluorescens* AF181 ability to invade epithelial cells and the possible mechanism involved.

Pathogenic microbes subvert normal host-cell processes to create a specialized niche, which enhances their survival. A common and recurring target of pathogens is the host cell's cytoskeleton, which is utilized by these microbes for purposes that include attachment, entry into cells, movement within and between cells, vacuole formation and remodelling. Our result demonstrated that A549 cells treatment with cytochalasin D resulted in significant decreases of both bacterial adherence and internalization. This result suggests that the cytoskeleton is implicated in *P. fluorescens* AF181 invasion. The bacterial adherence and internalization was also inhibited by

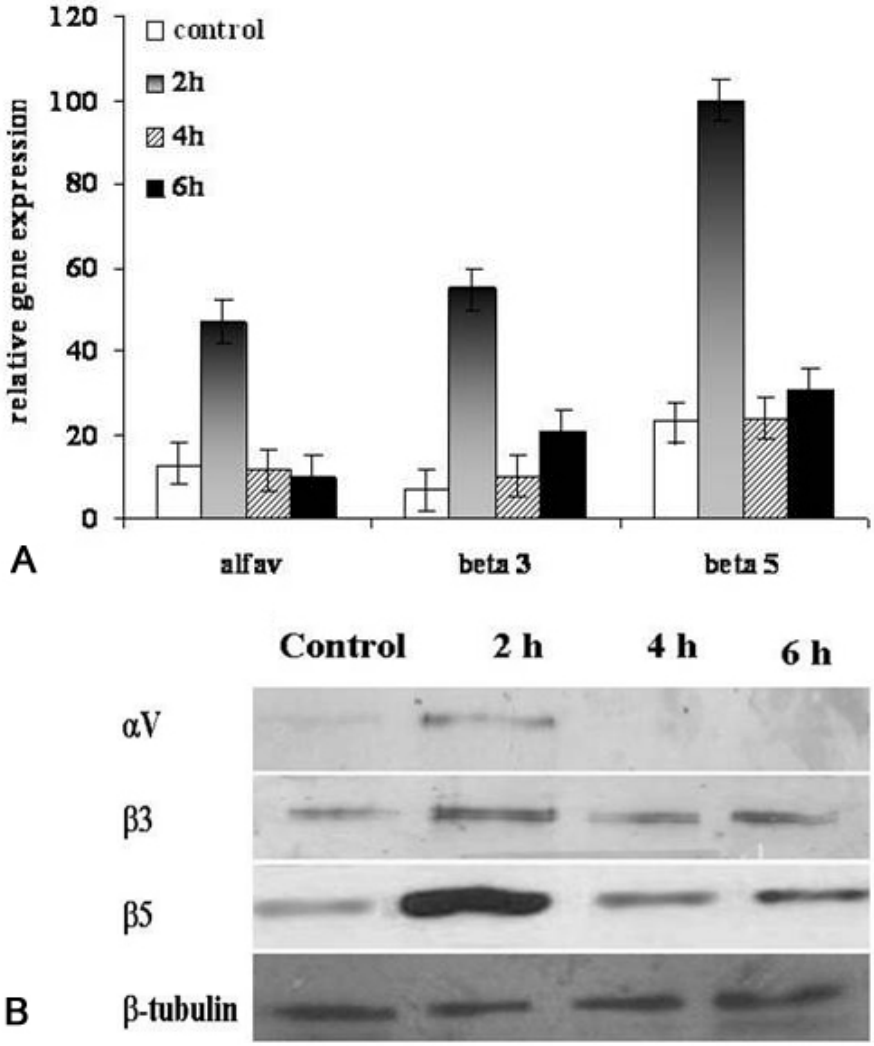

Figure 2. *P. fluorescens* up-regulates the alphav, beta3 and beta5 integrin in A549 cells. Real time quantitative PCR analysis using specific primers for alphav, beta3 and beta5 in *P. fluorescens* infected A549 cells. The data shown are representative of three different experiments ± S.D. Error bars represent standard. * P < 0.01, vs. control. (panel A). Western blot analysis for alphav, beta3 and beta5 content in *P. fluorescens* infected A549 cells at the indicated times. β–tubulin was used as internal control of protein load. (panel B).

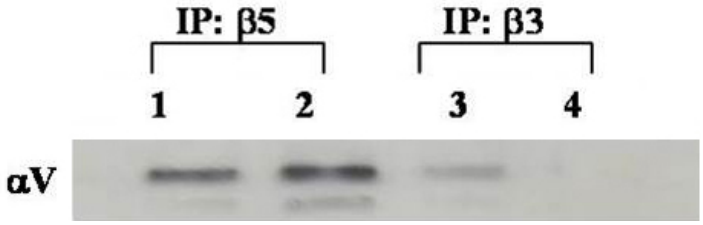

Figure 3. *P. fluorescens* up-regulates the alphavbeta5 integrin in A549 cells: four hundred micrograms of cell lysates from untreated and *P. fluorescens* infected A549 cells were immunoprecipitated with anti-beta3 and anti-beta5 antibodies and subjected to Western blot analysis for the alphav content (panel B). The data shown are representative of three different experiments.

genestein indicating that tyrosine kinases is involved in P. aeruginosa interaction with A549 cells. On the other hand it is known that the MAPK kinases pathway is also involved in microbial pathogenesis. Thus, the MAPK kinase

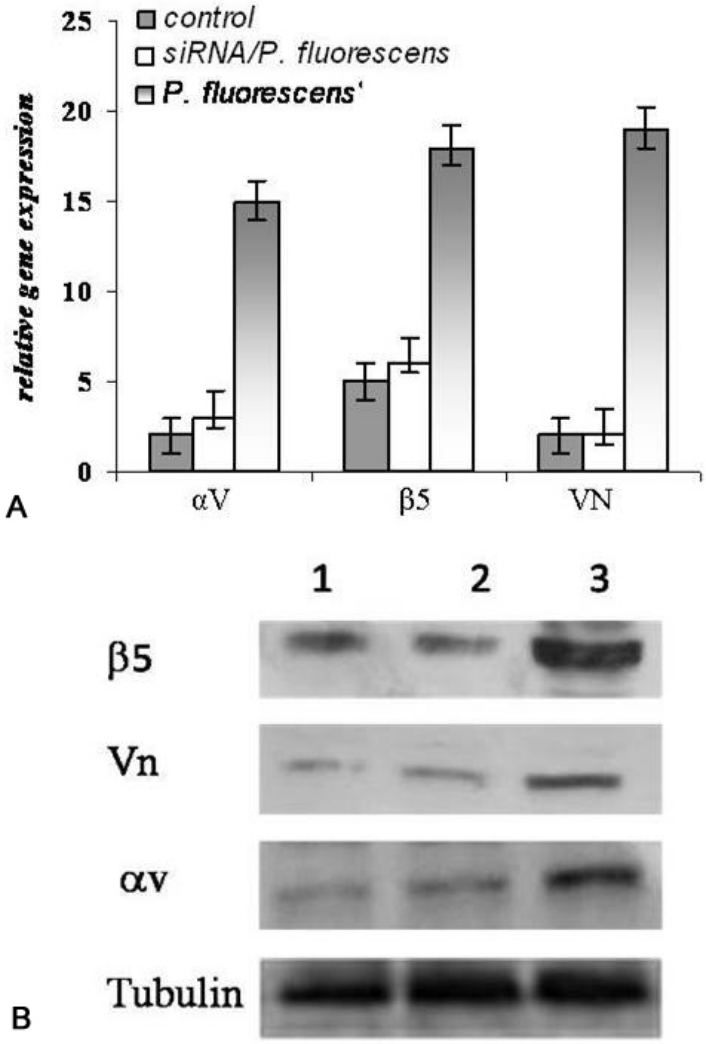

Figure 4. Real time quantitative PCR analysis using specific primers for alphav, beta5 and vitronectin in A549 cells after gene silencing. The data shown are representative of three different experiments ± S.D. Error bars represent standard. * $P < 0.01$, vs. control (panel A). Western blot analysis for alphav, beta5 and vitronectin content in A549 cells after gene silencing; line 1 control, line 2 siRNA/*P. fluorescens*, line 3 *P. fluorescens*. β–tubulin was used as internal control of protein load. (panel B). The data shown are representative of three different experiments.

inhibitor reduces invasion into some cell types by several organisms, including Chlamydia pneumoniae (22) and P. aeruginosa (20)

During the last decade, it has become clear that integrins significantly participate in various host-pathogen interactions involving pathogenic bacteria, fungi, and viruses. Several significant human pathogens are known to utilize integrins and exploit integrin-mediated signaling to invade various types of host cells. The resulting integrin-mediated signaling leads to tyrosine kinase phosphorylation, recruitment of adaptor molecules and cytoskeletal rearrangement required for bacterial engulfment, as well as induction of proinflammatory cellular responses. Such mechanisms can be advantageous to the microorganisms, because the invasion of host cells often confers protection against the immune response, and may facilitate microbial growth and spreading to other cells. In the present study, we focused on α5, αv, β3, and β5 subunits because they are involved in all ab integrin heterodimers expressed in lung epithelial cells (6). By immunoprecipitation experiments we have demonstrated that *P. fluorescens* AF181 up-regulates the αvβ5 expression to interact with A549 cells. In fact, the adherence to A549 cells and specific internalization was significantly decreased after silencing of αv and β5 integrin subunits.

Several pathogenic bacteria are able to bind integrin receptors directly, via some specific adhesins. These are typically not respiratory pathogens but ones that rather invade other mucosal tissues such as the

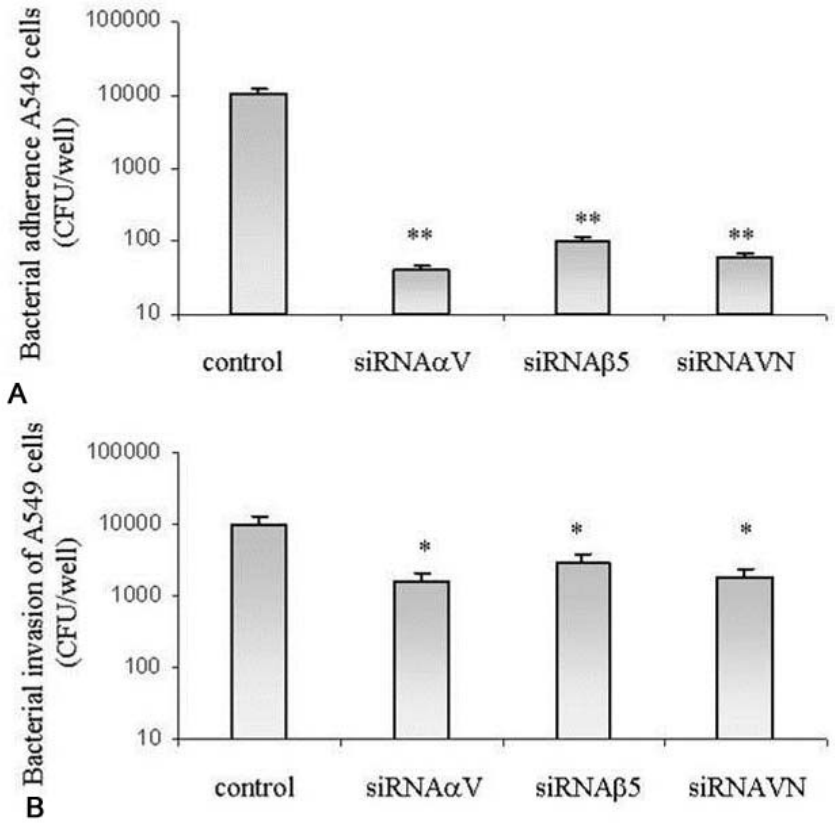

Figure 5. Effects of gene silencing of alphaV and beta5 integrins and their ligand VN, on *P. fluorescens* adherence and invasion. Semiconfluent cell cultures monolayers infected with *P. fluorescens* were incubated with antibodies for 3 h at 37 °C. Number of bacteria associated with A549 cells (panel A) and bacteria internalized (panel B) was determined by host cell lysis, plating, and counting CFU/well. Control: untreated cells. Assays were done in triplicate and repeated at least twice. Error bars represent standard deviations. * $P < 0.05$, ** $P < 0.05$, vs. control.

gastrointestinal epithelium (*Yersinia enterocolitica, Y. pseudotuberculosis and Helicobacter pylori*), or urethral epithelium (*Neisseria gonorrhoeae*) (23,24,25). However, the majority of integrin-binding microorganisms interact with integrins indirectly using ECM binding proteins as a molecular bridge to engage these receptors. In these cases, integrin receptors recognize the common arginine-glycine-aspartate (RGD) sequence that is present in ECM proteins, such as FN or VN (25,27) .

Our data demonstrated that *P. fluorescens* AF181 uses the αvβ5-VN pathway to interact with human non-phagocyte cells. Consequently, VN gene silencing strongly inhibited both adherence and invasion to A549 cells. In conclusion, our results provide evidence that αvβ5 integrins and its natural ligand VN is of major importance in *P. fluorescens* AF181 invasion of A549 respiratory cells *in vitro*.

6. ACKNOWLEDGEMENT

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Key Words: *Pseudomonas fluorescens,* Integrins, Vitronectin

Send correspondence to: Di Domenico Marina, Department of General Pathology, Second University of Naples, Via Costantinopoli, 16, 80100, Naples, Italy, Tel: 390815667557, Fax: 39081450169, E-mail: didomenico.marina@gmail.com