**Requirement of the Integrin β3 Subunit for Carcinoma Cell Spreading or Migration on Vitronectin and Fibrinogen**

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**Abstract.** FG human pancreatic carcinoma cells use integrin αvβ5 as their primary vitronectin receptor since they fail to express integrin αvβ3. These cells are unable to form focal contacts, spread, or migrate on vitronectin but readily do so on collagen in a β1 integrin-dependent manner. Transfection of FG cells with a cDNA encoding the integrin β3 subunit results in the surface expression of a functional integrin αvβ3 heterodimer providing these cells with novel adhesive and biological properties. Specifically, FG cells expressing β3 acquire the capacity to attach and spread on vitronectin as well as fibrinogen with β3 localization to focal contacts. Moreover, these cells gain the capacity to migrate through a porous membrane in response to either vitronectin or fibrinogen. These results demonstrate that the β3 and β5 integrin subunits when associated with αv, promote distinct cellular responses to a vitronectin extracellular environment.

**Materials and Methods**

**Cells and Cell Culture**

FG is a human pancreatic carcinoma cell line that fails to express mRNA for the β3 integrin subunit (Cheresh et al., 1989). FG-A and FG-B are two stably transfected sub-lines: FG-B, is transfected with a full length cDNA encoding human β3 gene; FG-A is a subline, transfected with vector alone. M21 human melanoma cells were a gift from Dr. Donald Morton (Department of Surgery, University of California, Los Angeles, CA). All cells were grown in RPMI 1640 with 10% FBS, 50 μg/ml gentamicin, and tested free from mycoplasma during these studies.
Antibodies
Integrin specific mAbs LM609 (αvβ3; Cheresh and Spiro, 1987), LM142 (αv; Cheresh and Harper, 1987), PM1C10 (β1; Carter et al., 1990); LM534 (β1; Wayner et al., 1991), and P3G2 (αvβ5; Wayner et al., 1991) were affinity purified from ascites on protein A-Sepharose (Affigel protein A, MAPS II Kit; Bio-Rad Laboratories, Richmond, CA). mAb AP3 was a generous gift from Dr. Peter Newman (Blood Center of Southwestern Wisconsin, Milwaukee, WI).

Adhesive Ligands
Vitronectin was prepared as described by Yatohgo et al. (1988). Fibrinogen was purified according to the method of Felting-Habermann et al. (1992). Collagen type I was purchased from Collaborative Research (New Bedford, MA).

cDNA Transfection of FG Cells
Full length cDNA encoding the human β3 integrin subunit, kindly provided by Dr. Larry Fitzgerald (University of Utah, Salt Lake City, UT) was ligated into the expression vector pCDNAneo (Invitrogen, San Diego, CA) and transfected into FG carcinoma cells using the lipofectin protocol (Calbiochem-Behring Corp., La Jolla, CA). Briefly, sub-confluent adherent cells were incubated with 2 µg cDNA and 40 µg lipofectin for 24 h, allowed to recover for 48 h before selection in neomycin for 2 wk at a concentration of 500 µg/ml. Neomycin-resistant cells were expanded and the cell surface expression of αvβ3 analyzed by flow cytometry using the αvβ3 complex-specific antibody, LM609. Immunoactive cells were enriched by four consecutive rounds of fluorescence-activated cell sorting, after which we observed stable surface expression of αvβ3 in ~40% of the transfected cell line. Positive (FG-B) and negative (FG-A) transfectant populations were recovered and maintained in vitro.

Cell Surface Labeling and Immunoprecipitation
Cell surface proteins were 125I-labeled using lactoperoxidase as previously described (Cheresh et al., 1989). Radiolabeled cells were lysed in RIPA buffer (10 mM Tris, pH 7.2, 150 mM NaCl, containing 1% Triton X-100, 1% deoxycholate, 0.1% SDS with 1% aprotinin (No. A6279; Sigma Chemical Co., St. Louis, MO) and 1 mM PMSF prior to immunoprecipitation with integrin-specific mAbs coupled to Sepharose (Pharmacia, Uppsala, Sweden) as described (Cheresh et al., 1989). Immunoprecipitates were analyzed by SDS-PAGE (Laemmli, 1970) under non-reducing conditions on 7.5% polyacrylamide gels and radiolabeled species visualized with autoradiography as previously described (Cheresh, 1987).

Adhesion Assay
Cell adhesion assays were performed as previously described with modifications (Wayner et al., 1991). In brief, sterile, untreated, bacteriological-grade polystyrene 48-well cluster plates (Costar, Cambridge, MA) were coated at 4°C overnight with adhesive ligands (200 µl at 10 µg/ml in PBS) and immediately before use blocked with 5% BSA in PBS, pH 7.4. Cells were harvested with trypsin/EDTA, washed once, and suspended in HBSS supplemented with 1% BSA, 1 mM CaCl2, 1 mM MgCl2, and 0.5 mM MnCl2 and then added to appropriate wells. Cells were permitted to adhere for various times at 37°C in 5% CO2 in the presence or absence of purified mAbs specific for various integrins (50 µg/ml) or the synthetic peptides (50 mM) GRGDS, PKDR, and SPGDRGK. Non-adherent cells were removed with gentle washing and cell attachment enumerated from photographs taken under phase contrast of quadruplicate, random fields from duplicate samples. Random non-specific adhesion was determined using the control protein BSA (20 µg/ml). Specific cell migration toward each matrix protein was calculated by subtracting BSA-mediated migration from total cell migration. In each case random migration <47% of total matrix-dependent migration.

Results
FG human carcinoma cells fail to express mRNA encoding the integrin β3 subunit (Cheresh et al., 1989). Thus we sought to determine the mechanism of FG cell attachment to vitronectin. FG cells were allowed to attach to vitronectin- or collagen-coated wells in the presence or absence of mAbs specific for integrins αvβ3, αvβ5, and β1. As shown in Table I, FG cell attachment to vitronectin can be significantly inhibited (67%) with mAb P3G2 directed to integrin αvβ5, while mAb LM609, directed to αvβ3, or mAb P4C10, directed to the β1 subunit, have little or no effect. These data indicate that αvβ5 is the primary vitronectin receptor expressed by FG cells. In contrast, mAbs LM609 and P3G2 have little or no effect on FG cell adhesion to collagen, whereas mAb P4C10 abolishes (91%) cell attachment on this substrate.

M21 human melanoma cells express αvβ3 as their primary vitronectin receptor (Wayner et al., 1991). Therefore, as shown in Table I, mAb LM609 significantly inhibited M21 cell adhesion to this matrix by 60%, whereas mAb P3G2, specific for αvβ5, had a reduced effect (28%). The β1-specific mAb, P4C10, had minimal effect on M21 cell attachment to vitronectin but completely inhibited attachment to collagen (97%), consistent with previous results (Strauss et al., 1989). These results collectively demonstrate that FG carcinoma cell adhesion to vitronectin is primarily mediated by αvβ5 while M21 melanoma cell attachment to this ligand primarily involves αvβ3.

To investigate the biological consequences of αvβ3- or αvβ5-dependent cell adhesion, we examined the capacity of FG cells or M21 cells to spread on vitronectin. As shown in Fig. 1, FG cells readily attach to, but do not spread on this matrix, even after 90 min. In contrast, these cells readily spread on a collagen matrix indicating that the intrinsic ability of FG cells to spread is not deficient. M21 human melanoma cells, which use primarily αvβ3 to attach to vitronectin-

rhodamine-conjugated goat anti-mouse IgG, or fluorescein-conjugated goat anti-rabbit IgG (Tago, Burlingame, CA). Focal adhesion plaques were visualized by the exclusion of vitronectin-specific antibody from the cell-substrate contacts as previously described (Wayner et al., 1991). Fluorescence was detected with a Photomicroscope (Carl Zeiss, Oberkochen, Germany) fitted with epifluorescence.

Cell Migration Assay
Cell migration assays were performed using modified Boyden chambers with a 6.5-mm-diam, 10-µm-thick, porous (80 µm) polystyrene membrane separating the two chambers (Transwell®, Costar). Soluble ligands (20 µg/ml) were placed in lower, migration chambers in 600 µl of serum-free RPMI 1640 supplemented with 1% ITS+ (Collaborative Research, New Bedford, MA), 50 µg/ml gentamycin, 2.5 µg/ml amphotericin B (Sigma Chemical Co.), and then incubated at 37°C for 30 min before the addition of cells. Sub-confluent 24-h cultures were harvested with trypsin/EDTA, washed once, and resuspended in the serum-free RPMI without adhesive ligands. Cells (50,000) were added in 100 µl to the upper chamber in the presence or absence of the synthetic peptides (50 mM) GRGDS, PKDR, or SPGDRGK, or mAbs (50 µg/ml) to various integrins. Migration is measured after 48-h incubation at 37°C by counting the number of cells recovered from the floor of the lower chamber. Non-adherent cellular debris is removed with gentle washing and cells fixed with 3% paraformaldehyde. Cell migration was quantitated from photographs taken under phase contrast of quadruplicate, random fields from duplicate samples. Random non-specific migration was determined using the control protein BSA (20 µg/ml). Specific cell migration toward each matrix protein was calculated by subtracting BSA-mediated migration from total cell migration. In each case random migration <47% of total matrix-dependent migration.
Table 1. Effects of Anti-Integrin mAbs on Cell Adhesion to Vitronectin or Collagen

| mAb    | FG cells Vitronectin | Collagen | M21 cells Vitronectin | Collagen |
|--------|----------------------|----------|-----------------------|----------|
| P3G2   | 67†                  | 2        | 28                    | 14       |
| (αvβ3) |                      |          |                       |          |
| LM609  | 0                    | 4        | 60                    | 5        |
| (αvβ3) |                      |          |                       |          |
| P4C10  | 22                   | 91       | 19                    | 97       |
| (β1)   |                      |          |                       |          |

* FG or M21 cells were allowed to attach to vitronectin- or collagen (10 μg/ml)-coated wells in the presence or absence of mAbs (50 μg/ml) for 60 min at 37°C as described in Materials and Methods. Standard deviations were routinely <10%.
† Represents percent inhibition of cell adhesion relative to adhesion in the absence of antibody.

expression of β3 in FG Cells Alters Their Biological Response to Vitronectin and Fibrinogen

To establish whether FG-B cells express the αvβ3 integrin in a functional form, cell adhesion assays were performed. FG-A and FG-B cells attach to vitronectin yet only FG-B cells can be inhibited with mAb LM609, directed to αvβ3, (55%, not shown). This adhesion event results in FG-B cell spreading on both vitronectin (Fig. 3, A-C) and fibrinogen (Fig. 3, B, left) within 60 min. In contrast, the β3 negative, parental FG cells (Fig. 1) or mock-transfected FG-A cells not only fail to spread on vitronectin but are unable to attach to fibrinogen (not shown). Moreover, FG-B cells which spread on both of these substrata, express β3 in focal contacts (Fig. 3, B and C) indicating that αvβ3 expression promotes the assembly of adhesion plaques and organization of the microspreading on this matrix (Fig. 1). These data suggest that the homologous integrins αvβ3 and αvβ5 mediate differential biological functions in response to a common ligand.

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Figure 2. SDS-PAGE analysis of ¹²⁵I-surface labeled FG-A (mock-transfected) and FG-B (β3-transfected) cells. FG carcinoma cells were transfected with a cDNA encoding full-length human β3 and neomycin-resistance genes. After drug selection for 2 wk, cells were sorted for αvβ3 expression by FACSs and stable sub-lines established. Cells were ¹²⁵I-surface labeled and extracted with detergent as described in Materials and Methods. Detergent extracts were immunoprecipitated with integrin-specific mAbs coupled to Sepharose beads. mAbs LM142 (αv, lane A), LM609 (αvβ3, lane B), P3G2 (αvβ5, lane C), and LM534 (β1, lane D).

Filament cytoskeleton leading to cell spreading on a vitronectin or fibrinogen matrix. Approximately 40% of the FG-B cell population express β3 and only these cells were found to spread on vitronectin or fibrinogen. In addition, mAb LM609 prevents cell spreading on vitronectin or fibrinogen providing further evidence for a role for αvβ3 in this process (data not shown).

To further investigate the biological role of integrin αvβ3 we examined the capacity of this receptor to potentiate FG-B cell migration. For these experiments we used modified Boyden chambers containing an 8.0 μm porous membrane. FG cells do not demonstrate significant or specific migration through the 8.0 μm pores separating these chambers in the presence of the control ligand BSA. However, both FG-A and FG-B cells readily migrate toward a collagen source yet only FG-B cells, expressing αvβ3, are capable of migrating toward vitronectin or fibrinogen (Fig. 4). FG-B cell migration to both vitronectin and fibrinogen is completely inhibited by mAb LM609 demonstrating that αvβ3 is responsible for this event (Fig. 4). In contrast, mAb P3G2, directed to αvβ5, had no effect on FG-A or FG-B cell migration to all substrata tested (data not shown), indicating this receptor fails to potentiate migration of these cells. In addition an RGD-containing synthetic peptide which functionally inhibits αvβ3-mediated cell attachment (Cheresh et al., 1989), completely blocks αvβ3-dependent migration (data not shown). It is of interest that the migration of αvβ3-expressing FG-B cells toward collagen is not significantly inhibited by mAb P4C10, directed to β1 integrins, suggesting an additional mechanism in these cells (Fig. 4). In fact, integrin αvβ3 has been reported, in some cells, to recognize collagen (Kramer et al., 1990) suggesting that, in addition to vitronectin and fibrinogen, αvβ3, when present, may also contribute to collagen-dependent cell migration.

Discussion

This study was designed to characterize the biological properties of two structurally and functionally related integrins αvβ3 and αvβ5 which are often expressed on the same cell.
Figure 3. Expression of β3 promotes FG-B cell attachment and spreading on vitronectin and fibrinogen with β3 localization to focal contacts. β3-Transfected FG-B cells were allowed to attach and spread on vitronectin-coated coverslips for 60 min. Non-adherent cells were removed with gentle washing and attached cells evaluated for spreading with phase-contrast microscopy (A). For fluorescence experiments FG-B cells were allowed to attach and spread on vitronectin- or fibrinogen-coated coverslips, fixed, permeabilized, and stained with mAb AP3 directed to the β3 integrin subunit, or anti-sera to vitronectin (B and C, respectively). β3 staining in cells spread on fibrinogen (B, left) and vitronectin (B, center and right) exactly co-distributes with focal contacts, visualized by the exclusion of vitronectin-specific antibody from the cell-substrate contacts as described under Materials and Methods (C, β3, left; anti-vitronectin, right; arrows, co-localized staining). Representative cells were photographed with a Zeiss microscope fitted with epifluorescence. Bars: (A) 25 μm; (B and C) 10 μm.

(Wayner et al., 1991). These receptors bind vitronectin in an RGD-dependent manner, contain identical α subunits and structurally similar β subunits (Cheresh and Spiro, 1987; Cheresh et al., 1989; McLean et al., 1990; Ramaswamy and Hemler, 1990; Suzuki et al., 1990). However, the cytoplasmic tails of β3 and β5 are structurally distinct thus raising the possibility that αvβ3 and αvβ5 mediate differential biological signals in response to vitronectin.

In this report we provide several lines of evidence that these integrins mediate distinct biological responses to a vitronectin substrate. Firstly, FG carcinoma cells use αvβ5 as their major vitronectin receptor since they fail to express β3 mRNA or protein (Cheresh et al., 1989) and attach but fail to spread on vitronectin and are incapable of attaching to fibrinogen. This is not because of a general deficiency in FG cell spreading since these cells readily attach and spread on collagen in an integrin β1-dependent manner. Secondly, M21 cells, which express αvβ3 as their major vitronectin receptor (Wayner et al., 1991), attach and spread on both vitronectin and fibrinogen with αvβ3 expressed in focal contacts at the end of actin filament bundles (Wayner et al., 1991). Thirdly, transfection of β3 negative FG cells with a cDNA encoding β3 results in the surface expression of αvβ3 which enables these cells to spread on vitronectin and fibrinogen. Moreover, β3 localizes to focal contacts on these transfected FG cells indicating that αvβ3 is directly involved in cell spreading on these matrix proteins. Finally, FG-B cells expressing the β3 gene product not only spread, but acquire the ability to migrate in response to vitronectin and fibrinogen. That this is because of the presence of αvβ3 on these cells is demonstrated by the ability of mAb LM609 (anti-αvβ3) or an RGD-containing peptide to block this migration. These results provide a rationale for the expression of αvβ3 and αvβ5 on the same cell where αvβ5 promotes simple adhesion while αvβ3 enables cells to modify their shape and mobility on vitronectin.

Although αvβ5 is the major vitronectin-binding integrin on FG cells we cannot completely exclude the possibility that additional vitronectin-binding integrins are expressed on these cells other than αvβ3. For example, integrin αvβ1 ap-
play a minor role in FG cell and M21 cell attachment to parently mediates vitronectin adhesion of certain cells (Bodary and McLean, 1990). It is conceivable that αβ1 could play a minor role in FG cell and M21 cell attachment to vitronectin since mAb P4C10 directed to β1 partially inhibited the attachment of both cell types to vitronectin (>20%). However, immunoprecipitation analysis failed to detect αβ1 on either cell type. In any event the expression of αβ1 or another vitronectin-binding integrin on FG cells does not account for measurable cell spreading and/or migration on vitronectin.

The cytoplasmic tail of the β5 subunit is structurally distinct from those of the β1 and β3 subunits (Ramaswamy and Hemler, 1990; McLean et al., 1990; Suzuki et al., 1990) and thus may be responsible for a distinct signal transduction event. Alternatively, it is conceivable that this subunit is simply incapable of interacting with one or more cytoplasmic proteins thought to be involved in the assembly of focal contacts. For example, talin and α-actinin, two proteins found in focal contacts, have been shown to directly bind integrins (Horowitz et al., 1986). In fact, α-actinin directly binds to a peptide derived from the cytoplasmic tail of β1 (Otey et al., 1990). Based on a mutational analysis of the β1 integrin it appears that the structural basis of integrin focal contact formation depends on three domains with the cytoplasmic tail of β1 (Reszka and Horowitz, 1992). It is noteworthy that β1 and β3 are extremely well conserved in each of these regions while β5 has virtually no sequence homology in the most COOH-terminal of these domains. It is conceivable that specific residues within this particular domain fail to support the localization of αβ5 into focal contacts. To test this hypothesis it will be necessary to examine the expression of truncated and/or chimeric integrin heterodimers in cells that normally fail to form focal contacts on a vitronectin substrate. It is also interesting to note that the cytoplasmic tail of β5 contains five serine residues not found in β1 or β3 (Ramaswamy and Hemler, 1990; McLean et al., 1990; Suzuki et al., 1990). Perhaps phosphorylation of one or more of these prevents the localization of αβ5 to focal contacts. In fact, the β subunit which may be related or identical to β5 becomes phosphorylated on serine in response to activators of protein kinase C (Freed et al., 1989).

The differential ability of αβ3 and αβ5 to promote cell spreading and migration may have profound biological implications during events associated with development, wound healing, and neoplasia where cell migration is known to take place. Thus, the genetic regulation of β3 and β5 may play a key role in determining the migratory status of a cell. Recent evidence supports this hypothesis. Non-differentiated keratinocytes, which are known to be migratory, express both αβ3 and αβ5 integrins while terminally differentiated keratinocytes, which do not migrate, no longer express αβ3 (Adams and Watt, 1991). Furthermore, the β3 subunit expressed by sub-confluent embryonic lung fibroblasts, is down regulated when these cells reach confluence (Bates et al., 1991). The role of αβ3 in melanoma cell migration may be very relevant for the metastatic phenotype of these cells. In fact, αβ3 was found to be preferentially expressed on metastatic and vertically invasive primary lesions whereas it was not detected on normal melanocytes, nevi, or horizontal primary melanoma (Albelda et al., 1990). The promiscuous ligand-binding capacity of αβ3 suggests that cells expressing this receptor can migrate on a wide variety of matrices and basement membranes. Our results demonstrate that fibrinogen a known ligand for αβ3 (Cheresh, 1987) can promote the migration of β3 transfected FG cells. Therefore the expression of αβ3 and the resulting phenotype may play an important role in a wide range of migratory events involving multiple biological phenomena.

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