The $\alpha_2\beta_1$ integrin mediates the malignant phenotype on type I collagen in pancreatic cancer cell lines

JJ Grzesiak¹ and M Bouvet*,¹

¹Department of Surgery (112-E), University of California, Veterans Affairs San Diego Healthcare System, 3350 La Jolla Village Drive, San Diego, CA 92161, USA

Pancreatic cancer is characterised by a hallmark desmoplastic response that includes upregulated expression of the extracellular matrix, and type I collagen in particular. Recent studies indicate that pancreatic cancer cells stimulate type I collagen synthesis in adjacent stellate cells, and that this upregulated type I collagen expression promotes the malignant phenotype in tumour cells as defined by increased proliferation, resistance to chemically induced apoptosis, and increased tumorigenesis. The integrin specificity of this interaction between type I collagen and tumour cells was not identified, however. In the present study, we examined eight pancreatic cancer cell lines for adhesion, proliferation, and migration, on types I and IV collagen, fibronectin, laminin, and vitronectin, as well as integrin expression. Our results indicate, for the overwhelming majority of cell lines, that type I collagen promotes the strongest adhesion, proliferation, and migration relative to the other substrates tested. Utilising function-blocking monoclonal antibodies directed against particular integrin subunits in cell adhesion and migration inhibition assays, we demonstrate further that the malignant phenotype on type I collagen is mediated specifically by the $\alpha_2\beta_1$ integrin. These results identify $\alpha_2\beta_1$ integrin-mediated adhesion to type I collagen as a potential therapeutic target in the treatment of pancreatic cancer.

Keywords: laminin; fibronectin; extracellular matrix; migration; adhesion

Pancreatic ductal adenocarcinoma is thought to result from a progressive accumulation of mutations in such genes as K-ras, CDKN2A, p53, BRCa2, p16ink, and SMAD4 (Bardeesy and DePinho, 2002). The SMAD4 mutations, in particular, which result in the constitutive activation of transforming growth factor $\beta_1$ (TGF$\beta_1$) signalling, are generally considered to be responsible for the hallmark desmoplastic response, which includes upregulated expression of the extracellular matrix (ECM), and type I collagen in particular (Mollenhauer et al., 1987; Lohr et al., 1994; Shimoyama et al., 1995; Linder et al., 2001; Bardeesy and DePinho, 2002; Menke and Adler, 2002; Tempia-Caliera et al., 2002; Iacobuzio-Donahue et al., 2003a, b).

We have previously shown that $\alpha_2\beta_1$ integrin-mediated adhesion on type I collagen promotes the malignant phenotype in FG pancreatic cancer cells, as defined by increased proliferation and haptokinetic cell migration, downregulated expression and localisation of E-cadherin and $\beta$-catenin in cell–cell contacts, increased phosphorylation of GSK3 and PKB/Akt, and increased expression of PTHrP, IL-6, and IL-8 compared to fibronectin (Menke et al, 2001). Recently, we extended our initial observations in FG cells by demonstrating similar phenotypic differences in BxPC-3, Colo-357, and CFPAC cells on type I collagen compared to fibronectin; that is, increased haptokinetic cell migration, downregulated expression and localisation of E-cadherin and $\beta$-catenin in disrupted cell–cell contacts, increased phosphorylation of GSK3 and PKB/Akt, and decreased expression of PTHrP, IL-6, and IL-8. Furthermore, functional studies with pharmacological inhibitors for GSK3 and PKB/Akt suggest that these signalling effectors are involved in the mechanism of $\alpha_2\beta_1$ integrin-mediated regulation of the malignant phenotype in FG cells (Grzesiak et al., 2006).

Recent studies from several other laboratories have also converged on the hypothesis that type I collagen, in particular, plays an active role in vitro and in vivo in the pathophysiology of pancreatic cancer (Armstrong et al, 2004; Bachem et al, 2005). Specifically, these studies demonstrated in vitro that pancreatic cancer cell lines stimulated the production of type I collagen from adjacent stellate cells, which resulted in increased cancer cell proliferation and resistance to chemically induced apoptosis. In vivo, these studies also showed that type I collagen expression was closely associated with both the cancer and stellate cells, and that introduction of stellate cells along with the tumour cells increased tumorigenesis in nude mice. The integrin specificity of this interaction between tumour cells and type I collagen was not identified, however.

In the present study, we surveyed a broad range of pancreatic cancer cell lines from various tumour grades, origins, and genetic
aberrations, for relative integrin expression, as well as adhesion, proliferation, and haptokinetic migration on types I and IV collagen, fibronecin, laminin, and vitronectin. Our results indicate strong $\alpha_2\beta_1$ integrin-mediated type I collagen adhesion, proliferation, and migration for all cell lines, except MiaPaCa-2, which does not express the $\alpha_2\beta_1$ collagen-binding integrin. These results collectively suggest an active and dynamic role for the ECM, and type I collagen in particular, in the growth, progression, and metastasis of pancreatic cancer, and identify the $\alpha_2\beta_1$ integrin as a potential therapeutic target in the treatment of this devastating disease.

MATERIALS AND METHODS

 Cells
Seven human pancreatic adenocarcinoma cell lines, Capan-1, CFPAC, Colo-357, AsPC-1, BxPC-3, MiaPaCa-2, and Panc-1 were obtained from the American Type Culture Collection (Rockville, MD, USA). FG cells are a fast-growing, metastatic variant of the Colo-357 cell line (Vezedis et al., 1990), and were the generous gift from Dr S Silletti. The cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum (FBS) in a humidified atmosphere containing 5% CO$_2$ at 37°C.

 Antibodies
The function-blocking monoclonal antibodies directed against particular integrin subunits and integrins used in these studies have been described (Cheresh and Spiro, 1987; Wayner and Carter, 1987; Sonnenberg et al., 1998; Wayner et al., 1988, 1993; Fabbri et al., 1996), and include FB12 ($\alpha_2$), P1E6 ($\alpha_2$), P1B5 ($\alpha_5$), P1D6 ($\alpha_5$), GoH3 ($\alpha_5$), P5D2 ($\beta_1$), LM609 ($\alpha_5$, $\beta_1$), P1F6 ($\alpha_5$, $\beta_3$), and ASC-3 ($\beta_3$) (Chemicon International, Temecula, CA, USA). For immunoprecipitation (IP) experiments, we used the monoclonal antibodies described above along with rabbit polyclonal antisera against the $\alpha_5$ integrin subunit, which has also been described previously (Ruoslahti and Pierschbacher, 1987).

Cell adhesion assays
Cell adhesion assays were performed as described previously (Ruoslahti et al., 1982). Briefly, $5 \times 10^4$ cells, serum-starved 24 h prior to assay, in 100 µl of serum-free DMEM supplemented with 1 mg ml$^{-1}$ bovine serum albumin (BSA) were added to each well of nontissue culture-treated 96-well microtitre plates (Becton Dickinson, Franklin Lakes, NJ, USA) that were coated with either type I collagen, type IV collagen, fibronecin, laminin, or vitronectin, each at 5 µg ml$^{-1}$, were then placed on top of the lower chambers, and the upper chambers were secured in place. Upper chambers were filled with 5 × 10$^4$ FG, AsPC-1, MiaPaCa-2, BxPC-3, or CFPAC cells that were serum-starved 24 h prior to assay, in the same medium described above. Lower chamber final volumes were 30 µl and the upper chambers were 50 µl. The entire apparatus was then incubated for 24 h at 37°C. After the incubation period, the filters were fixed in methanol and stained with 0.5% toluidine blue in 3.7% formaldehyde. Excess stain was washed away with water, the attached cells on the upper side of the filters were mechanically removed using wet, cotton-tipped applicators, and the migratory cells on the underside of the filters were quantitated by counting four high-powered fields ($\times$ 100 magnification) per well using an inverted light microscope (Olympus BH 2).

Inhibition of cell migration assays
In some experiments, migration assays were conducted as described above, with the addition of function-blocking monoclonal antibodies directed against specific integrin subunits to the upper chamber at the time cells were added. Final antibody concentrations were 50 µg ml$^{-1}$ for FB12 ($\alpha_2$), P1E6 ($\alpha_2$), and P1B5 ($\alpha_5$), and 25 µg ml$^{-1}$ for P5D2 ($\beta_1$). Type I collagen-coating concentrations were 5 µg ml$^{-1}$ for each cell line. Cells ($5 \times 10^4$) were added to each well, except for BxPC-3 cells, which were used at 2.5 × 10$^4$ cells well$^{-1}$.

Immunoprecipitations
To perform IPs, antibodies (4 µg) were adsorbed for 2 h at room temperature onto 25 µl packed anti-mouse IgG–agarose, anti-rat IgG–agarose or protein A–sepharose (Sigma, St Louis, MO, USA) in IP wash buffer (50 mM Tris-Hcl, pH 7.5, 0.5 mM NaCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, and 0.1% Tween-20). Antibody-coated beads were washed and 400 µg protein from cell-surface-biotinylated cell lysates was added in IP wash buffer. Protein content was determined using the modified Bradford reagent according to the manufacturer’s instructions (BioRad, Hercules, CA, USA). Pancreatic cancer cell lines were cell surface biotinylated using EZ-Link™ Sulfo-NHS-LC-Biotin (sulfosuccinimidyl-6-(biotinamido) hexanoate) according to the manufacturer’s instructions (Pierce Biotechnology Inc., Rockford, IL, USA) as described previously (Grzesiak et al., 2005b). Cell lysates were prepared as described previously, except ethylenediaminetetraacetic acid was removed and replaced with 1 mM CaCl$_2$ and 1 mM MgCl$_2$ (Grzesiak et al., 2004, 2005a, b). After incubation overnight at 4°C, the beads were washed six times with IP wash buffer, 50 µl 2 × NU–PAGE buffer sample buffer was added (Invitrogen, Carlsbad, CA, USA), samples were incubated 15 min at 70°C, and 25 µl of sample was carefully loaded, separated on 12% NU–PAGE gels under nonreducing conditions with 1 mg ml$^{-1}$ BSA. Purified monoclonal antibodies were added at a final concentration of 25 µg ml$^{-1}$ in the serum-free medium described above. After 45 min at 37°C, media were removed; attached cells were fixed, stained, destained, solubilised, and quantified as described above.

Migration assays
Migration assays were conducted using the modified Boyden chamber as described previously (Grzesiak et al., 1992, 2005a). Briefly, the chamber consists of two compartments separated by a filter, and migration was measured by counting the number of cells crossing the membrane through pores of defined size. Lower chambers were filled with serum-free DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 1 mg ml$^{-1}$ BSA. Porcine polycarbonate membrane filters (8 µm) (Neuro Probe Inc., Gaithersburg, MD, USA) that were coated with either type I collagen, type IV collagen, fibronecin, laminin, or vitronectin, each at 5 µg ml$^{-1}$, were then placed on top of the lower chambers, and the upper chambers were secured in place. Upper chambers were filled with 5 × 10$^4$ FG, AsPC-1, MiaPaCa-2, BxPC-3, or CFPAC cells that were serum-starved 24 h prior to assay, in the same medium described above. Lower chamber final volumes were 30 µl and the upper chambers were 50 µl. The entire apparatus was then incubated for 24 h at 37°C. After the incubation period, the filters were fixed in methanol and stained with 0.5% toluidine blue in 3.7% formaldehyde. Excess stain was washed away with water, the attached cells on the upper side of the filters were mechanically removed using wet, cotton-tipped applicators, and the migratory cells on the underside of the filters were quantitated by counting four high-powered fields ($\times$ 100 magnification) per well using an inverted light microscope (Olympus BH 2).
conditions, and transferred to nitrocellulose. After blocking with 3% BSA in PBS/0.1% Tween-20 overnight at 4 °C, membranes were incubated with horseradish peroxidase-conjugated streptavidin (1:25 000 dilution; Pierce Biotechnology Inc.) for 30 min at room temperature, followed by washing, and detection of peroxidase activity using chemiluminescence according to the manufacturer’s instructions (Amersham Biosciences, Little Chalfont, UK).

**Proliferation assays**

The 96-well polystyrene culture dishes, not treated for tissue culture, were coated with type I collagen, type IV collagen, fibronectin, laminin, or vitronectin at the following concentrations: for AsPC-1 cells – types I and IV collagen at 5 μg ml⁻¹, fibronectin at 25 μg ml⁻¹, laminin at 15 μg ml⁻¹, and vitronectin at 5 μg ml⁻¹; for CFPAC cells – types I and IV collagen at 5 μg ml⁻¹, fibronectin and laminin at 25 μg ml⁻¹, and vitronectin at 10 μg ml⁻¹; for BxPC-3 cells – types I and IV collagen at 5 μg ml⁻¹, and fibronectin, laminin, and vitronectin each at 15 μg ml⁻¹; for MiaPaCa-2 cells – types I and IV collagen at 10 μg ml⁻¹, fibronectin at 25 μg ml⁻¹, laminin at 15 μg ml⁻¹, and vitronectin at 10 μg ml⁻¹; and for FG cells – types I and IV collagen at 5 μg ml⁻¹, laminin at 10 μg ml⁻¹, fibronectin at 25 μg ml⁻¹, and vitronectin at 10 μg ml⁻¹. These coating concentrations promote maximal adhesion for each cell line as shown in Figure 1. Twenty-four hour, serum-starved pancreatic cancer cells (5 × 10⁴ well⁻¹) were cultured under serum-free conditions on these ECM substrata over a 4-day time course. At the indicated time points, triplicate proliferation determinations were quantified by measuring the absorbance at 450 nm and subtracting the value obtained for each cell line on each ECM substrate at initial seeding using CellTiter 96 Aqueous One Solution Cell Proliferation Assay reagent according to the manufacturer’s instructions (Promega, Madison, WI, USA). This reagent is composed of a novel tetrazolium compound (methyl-p-tolyl sulfide), and an electron coupling reagent, phenazine ethosulphate.

**Statistics**

Statistical significance (P < 0.05) was determined using two-tailed Student’s t-tests.

**RESULTS**

Type I collagen promotes maximal cell adhesion in pancreatic cancer cell lines

We first tested the panel of commonly used pancreatic cancer cell lines shown in Table 1, which covers all tumour grades and genetic mutations, and includes cell lines derived from primary tumours, ascites, and liver and lymph node metastases, in serum-free adhesion assays on the stromal ECM proteins, type I collagen, fibronectin, and vitronectin, and the basement membrane proteins, type IV collagen and laminin. Figure 1 demonstrates that in seven of eight cell lines tested, type I collagen promoted the strongest cell attachment relative to the other ECM proteins, and did so at much lower concentrations compared to the other ECM proteins tested. Only MiaPaCa-2 cells did not attach to type I collagen, nor did they attach to type IV collagen, even at the highest coating concentrations. Attachment to fibronectin was also quite strong in all the cell lines, except for the grade 1 tumour cell...
lines, Capan-1 and CFPAC, and the grade 2 cell line, AsPC-1, which showed only weak to moderate attachment. Laminin adhesion was moderate to weak in all cell lines tested. Adhesion of pancreatic cancer cell lines on vitronectin was the most variable. Relative to the other ECM proteins, FG, Colo-357, BxPC-3, and CFPAC cell adhesion to vitronectin was limited, while AsPC-1, Capan-1, Panc-1, and MiaPaCa-2 adhesion to vitronectin was very strong. A trend towards increased vitronectin adhesion with increasing tumour grade was apparent, with both grade 3-derived cell lines, MiaPaCa-2 and Panc-1, attaching strongly to vitronectin. Relative to the other cell lines, BxPC-3, CFPAC, and Panc-1 exhibited the strongest adhesion, regardless of substrate (unpublished observations).

Table 1

| Cell line | Origin       | Grade | K-ras p53 | p16INK4A | DPC4/Smad4 |
|-----------|--------------|-------|-----------|----------|------------|
| Capan-1   | Liver metastasis | 1     | mut       | mut      | mut        |
| CFPAC     | Liver metastasis | 1     | mut       | mut      | mut        |
| Colo-357  | Lymph node metastasis | 2     | mut       | w.t.     | mut        |
| FG        | Lymph node metastasis | 2     | mut       | w.t.     | mut        |
| AsPC-1    | Ascites      | 2     | mut       | Mut      | w.t.       |
| BxPC-3    | Primary      | 2     | w.t.      | mut      | mut        |
| MiaPaCa-2 | Primary      | 3     | mut       | mut      | w.t.       |
| Panc-1    | Primary      | 3     | mut       | mut      | Mut        |

mut = mutated; w.t. = wild type.

Type I collagen promotes maximal proliferation of pancreatic cancer cell lines

We next examined the differential effect of the ECM on pancreatic cancer cell proliferation. Using ECM protein concentrations that promoted maximal cell adhesion for each of the substrates with five representative cell lines still representing all three tumour grades, we conducted 96 h proliferation assay time-course studies under serum-free conditions. All cell lines tested grew well throughout the 4-day time course, even in the absence of serum, as long as the cells were given an ECM substrate to which they could attach. Figure 2 shows a strong direct correlation between pancreatic cancer cell adhesion and proliferation, with AsPC-1, BxPC-3, CFPAC, and FG cells demonstrating maximal proliferation on type I collagen relative to the other ECM proteins examined. MiaPaCa-2 cells, by contrast, showed essentially no proliferation on either type I or type IV collagen. Visual inspection confirmed that MiaPaCa-2 cells were found as nonadherent floating aggregates when cultured on either of the collagens (unpublished observations).

Figure 2

Type I collagen promotes maximal pancreatic cancer cell proliferation. The 96-well polystyrene culture dishes, not treated for tissue culture, were coated with type I collagen, type IV collagen, fibronectin, laminin, or vitronectin as described in Materials and Methods. Twenty-four hour, serum-starved MiaPaCa-2, AsPC-1, BxPC-3, CFPAC, and FG cells (5 × 10^4 well^-1) were cultured under serum-free conditions on the indicated ECM substrates over a 4-day time course. At the indicated time points, triplicate determinations were quantified for each cell line on each substrate by measuring the absorbance at 450nm and subtracting the value obtained at initial seeding (T = 0) using CellTiter 96 Aqueous One Solution Cell Proliferation Assay reagent according to the manufacturer’s instructions (Promega, Madison, WI, USA). Data presented represent the mean ± s.e.m. from two independent experiments.
Type I collagen promotes maximal haptokinetic cell migration in pancreatic cancer cell lines

We next examined all of our cell lines for haptokinetic cell migration on the ECM. As opposed to chemotaxis, where cell migration is stimulated by a growth factor gradient, haptokinetic migration reflects the natural ability of the substrate to promote cell movement. Figure 3 demonstrates that type I collagen promotes maximal haptokinesis in all the SMAD4-mutated cell lines, including Capan-1, CFPAC, BxPC-3, Colo-357, and FG, cells. Interestingly, MiaPaCa-2, AsPC-1, and Panc-1 cells, which exhibit wild-type SMAD4, and exhibit only moderate cell adhesion on laminin (Figure 1), migrated maximally on that substrate. Panc-1 and AsPC-1 cells also showed significant migration on type I collagen, whereas MiaPaCa-2 cells exhibited no haptokinesis on the substrate. Vitronectin and fibronectin promoted relatively weak haptokinesis on all cell lines tested, although the two substrates promoted very strong cell adhesion in some cases (Figure 1). Relative to the other cell lines tested, BxPC-3 cells exhibited the most haptokinesis followed by Panc-1 and CFPAC, regardless of substrate (unpublished observations). Taken together, these results demonstrate that type I collagen promotes a phenotype that is consistent with malignancy in the majority of our pancreatic cancer cell lines, as defined by maximal cell adhesion, proliferation, and migration, relative to the other ECM proteins tested. These data also identify laminin as a strong migratory substrate in those cell lines lacking an SMAD4 mutation (AsPC-1, MiaPaCa-2, and Panc-1).

The collagen-binding, $\alpha_2\beta_1$ integrin mediates the malignant phenotype in pancreatic cancer cell lines

Integrins mediate the adhesion of cells to the ECM (Ruoslahti et al, 1994), and we have recently demonstrated in a limited study with FG cells that the $\alpha_2\beta_1$ integrin mediates type I collagen adhesion and proliferation (Grzesiak et al, 2004, 2005a). Presently, we conducted IPs of all eight cell lines for relative expression of the $\alpha_1$, $\alpha_2$, $\alpha_5$, $\alpha_6$, $\beta_1$, and $\beta_4$ integrin subunits, and the $\alpha_2\beta_3$ and $\alpha_6\beta_3$ integrins. Figure 4 demonstrates that the $\alpha_2$ integrin subunit is expressed by all of our pancreatic cancer cell lines, except for MiaPaCa-2 cells, which do not attach, proliferate, or migrate on types I or IV collagen under serum-free conditions (Figures 1–3). Interestingly, Figure 4 also demonstrates that the collagen-binding $\alpha_2$ integrin subunit is expressed in all cell lines with TGFβ1-activating SMAD4 mutations, including Capan-1, CFPAC, Colo-357, FG, and BxPC-3, but not in those cells expressing wild-type SMAD4, including AsPC-1, MiaPaCa-2, and Panc-1. All cell lines appear to be immunoreactive to varying degrees for the $\alpha_3$, $\alpha_5$, $\alpha_6$, $\beta_1$, and $\beta_4$ integrin subunits, as well as the $\alpha_2\beta_3$ and $\alpha_6\beta_3$ integrins. These integrin expression results are generally consistent with relative strength of adhesion and migration, BxPC-3, CFPAC, and

![Figure 3](image-url)  
Type I collagen promotes maximal pancreatic cancer cell migration. Pore polycarbonate membrane filters (8 μm) were coated with either type I collagen, type IV collagen, fibronectin, laminin, or vitronectin, at 5 μg ml⁻¹. Lower chambers of the modified Boyden chamber were filled with 30 μl well⁻¹ of serum-free DMEM supplemented with 1 mg ml⁻¹ BSA, the coated filters were then placed on top of the lower chambers, and the upper chambers were secured in place. Upper chambers were filled with 5 × 10⁵ FG, AsPC-1, MiaPaCa-2, BxPC-3, or CFPAC cells that were serum-starved 24 h prior to assay, in 50 μl of the same media described above. The entire apparatus was then incubated for 24 h at 37°C. After the incubation period, the filters were fixed in methanol and stained with 0.5% toluidine blue in 3.7% formaldehyde. Excess stain was washed away with water, the attached cells on the upper side of the filters were mechanically removed using wet, cotton-tipped applicators, and the migratory cells on the underside of the filters were quantitated by counting four high-powered fields (×100 magnification) per well using an inverted light microscope (Olympus BH 2). Results presented represent the mean ± s.e.m. from two independent experiments with at least three replicates per substrate for each cell line. 100% = maximal cell number for each cell line and each set of ECM proteins.
Panc-1 cells demonstrate the strongest overall adhesion, migration, and integrin expression.

As collagen-binding integrins have been shown to include \( \alpha_1 \beta_1 \), \( \alpha_2 \beta_1 \), and \( \alpha_5 \beta_1 \), depending on the cell type (Ruoslahti, 1991), and because these integrins are variously expressed by all of our cell lines, we conducted inhibition of adhesion and migration assays on type I collagen using function-blocking monoclonal antibodies directed against specific integrin subunits. Figure 5A and B demonstrate clearly that pancreatic cancer cell adhesion and migration on type I collagen is mediated exclusively by the \( \alpha_2 \beta_1 \) integrin, and that neither the \( \alpha_1 \), \( \alpha_3 \), \( \alpha_5 \), or \( \alpha_6 \) integrin subunits, nor the \( \alpha_2 \beta_3 \) or \( \alpha_5 \beta_3 \) integrins appear to be involved in the adhesive interactions between AsPC-1, BxPC-3, CFPAC, or FG pancreatic cancer cells and type I collagen. Immunoprecipitation studies shown in Figure 4 and previous studies by us and other laboratories (Cheresh and Spiro, 1987; Wayner and Carter, 1987; Sonnenberg et al., 1988; Wayner et al., 1988, 1993; Grzesiak et al., 1992, 2005a; Fabbri et al., 1996) demonstrate that the monoclonal antibodies used for the type I collagen inhibition studies are functional.

DISCUSSION

In the present study, we characterised the adhesion, proliferation, and haptokinetic cell migration, as well as the relative integrin expression profiles of eight pancreatic cancer cell lines comprised of all three tumour grades, and derived from primary tumours, liver and lymph node metastases, and ascites. We tested physiologically relevant ECM components from the stroma, including type I collagen, fibronectin, and vitronectin, as well as the basement membrane proteins, type IV collagen and laminin, for their ability to promote a malignant phenotype in pancreatic cancer cell lines, as defined by increased adhesion, proliferation, and migration.

The clear indication that most of our pancreatic cancer cell lines attach, proliferate, and migrate maximally on type I collagen compared to the other ECM proteins examined is not surprising, in that the hallmark desmoplastic reaction associated with pancreatic adenocarcinoma, including upregulated type I collagen expression, has been appreciated for some time now (Mollenhauer et al., 1987; Lohr et al., 1994; Shimoyama et al., 1995; Tani et al., 1997; Kuehn et al., 1999; Linder et al., 2001; Tempia-Caliera et al., 2002). Recent analyses using microarray and serial analysis of gene expression technology confirm those studies and point out the strong expression of many other ECM genes in pancreatic cancer as well (Iacobuzio-Donahue et al., 2003a,b; Binkley et al., 2004; Stein et al., 2004).

There is growing evidence that the desmoplastic response associated with pancreatic cancer is representative of dysregulated normal injury repair processes that include TGF\(\beta_1\)-stimulated expression of type I collagen and other ECM proteins (Menke and Adler, 2002). In fact, it has been shown in vitro that TGF\(\beta_1\) upregulates type I collagen expression in pancreatic cancer (Lohr et al., 2001). Very recent studies from two independent laboratories also provide supporting data that type I collagen, in particular, plays an active role in vitro and in vivo in the pathophysiology of pancreatic cancer (Armstrong et al., 2004; Bachem et al., 2005).

Specifically, these studies demonstrated in vitro that pancreatic cancer cell lines stimulated the production of type I collagen from adjacent stellate cells, which resulted in increased cancer cell proliferation and resistance to chemically induced apoptosis. In vivo, these studies also showed that type I collagen expression was closely associated with both the cancer and stellate cells, and that introduction of stellate cells along with tumour cells increased tumorigenesis in nude mice. Our present results are the first to indicate that even though there are several potential integrin receptors expressed by our pancreatic cancer cell lines, the \( \alpha_2 \beta_1 \) integrin exclusively mediates type I collagen adhesion, proliferation, and migration.

The previous demonstrations that type I collagen is clearly upregulated (Mollenhauer et al., 1987; Lohr et al., 1994; Shimoyama et al., 1995; Tani et al., 1997; Kuehn et al., 1999; Linder et al., 2001; Tempia-Caliera et al., 2002; Iacobuzio-Donahue et al., 2003a,b; Binkley et al., 2004; Stein et al., 2004), and mediates a malignant phenotype in pancreatic cancer in vivo (Armstrong et al., 2004; Bachem et al., 2005), correlates directly with our present in vitro observations, and are further extended by our demonstration that the \( \alpha_2 \beta_1 \) integrin specifically regulates this type I collagen-mediated phenotype in multiple pancreatic cancer cell lines. These observations collectively suggest that targeting the \( \alpha_2 \beta_1 \) integrin may have therapeutic value in the treatment of pancreatic cancer in vivo. In support of the inhibition of the \( \alpha_2 \beta_1 \) integrin with function-blocking monoclonal antibodies inhibited human osteosarcoma cell (HOS) tumour growth in SCID mice (Miura et al., 2005). In another study, OCUM-2MD3, a human squamous gastric carcinoma cell line overexpressing \( \beta_1 \) and \( \beta_1 \) integrins, produced peritoneal dissemination in nude mice compared to control cells, and its invasive ability was significantly decreased following the addition of \( \beta_1 \) or \( \beta_1 \) integrin antibodies (Nishimura et al., 1996).

Our present results also identify two distinct and previously unknown phenotypes that correlate directly with the presence or absence of TGF\(\beta_1\)-activating SMAD4 mutations (see Table 1). In the five pancreatic cancer cell lines with SMAD4 mutations, including Capan-1, CFPAC, Colo-357, FG, and BxPC-3, type I collagen promotes maximal adhesion, migration, and proliferation. Regarding integrin expression, these same five cell lines express the collagen-binding \( \alpha_2 \beta_1 \) integrin. In the three cell lines expressing wild-type SMAD4, including AsPC-1, MiaPaCa-2, and Panc-1, maximal migration occurs on laminin and there is a distinct absence of the \( \alpha_2 \beta_1 \) integrin. These results are in agreement with previous results indicating that Panc-1 cells are negative and that BxPC-3 cells are positive for the \( \alpha_1 \) integrin subunit by FACS analyses (Lohr et al., 1996). It is intriguing that TGF\(\beta_1\) treatment of the NR4 hepatocyte cell line increased the expression of \( \alpha_1 \) integrin subunit mRNA (Serra et al., 1994), directly correlating with our findings that cell lines with TGF\(\beta_1\)-activating SMAD4 mutations express the \( \alpha_2 \beta_1 \) integrin. Interestingly, while the \( \alpha_2 \beta_1 \) integrin is only expressed in cell lines
containing SMAD4 mutations, the function-blocking monoclonal antibody against z1 had no effect on pancreatic cancer cell adhesion or migration on type I collagen either alone or in combination with the z2 function-blocking monoclonal antibody. Our data indicate that only the z2β1 integrin mediates type I collagen adhesion, migration, and proliferation in pancreatic cancer cell lines.

Immunoprecipitation results indicate that seven of the eight pancreatic cancer cell lines express the collagen-binding z2β1 integrin. While expression of the z2 integrin subunit has been demonstrated previously for most of these cell lines (Lehr et al., 1996; Sawai et al., 2001; Miyamoto et al., 2004), to our knowledge, this is the first demonstration that the CFPAC cell line is also z2 integrin subunit positive. These results are in agreement with reports using other pancreatic cancer cell lines, including PC-2,-3, and -44, PaTu 8988S, HPAF, Capan-2, PaTu 8902, and PaTu-2 cells, which also demonstrated type I collagen adhesion and z2 integrin subunit expression (Mollenhauer et al., 1987; Weinel et al., 1992).
Our present results extend those previous studies by demonstrating, in multiple cell lines, the relative strength of type I collagen adhesion, proliferation, and migration compared to the other physiologically relevant ECM proteins tested in these studies. These results also rule out a contribution from the $\alpha_2\beta_1$ integrin in mediating tumour cell adhesion to type I collagen in pancreatic cancer.

In agreement with previous studies, our results also indicate that MiaPaCa-2 cells do not attach to types I or IV collagen, and they do not express the $\alpha_2$ integrin subunit (Sawai et al, 2001; Miyamoto et al, 2004). In fact, it has been recently shown that attempts to grow MiaPaCa-2 cells on type I collagen substrates resulted in apoptosis (Vaquero et al, 2003). Our results extend these early findings by demonstrating for the first time that MiaPaCa-2 cells are negative for $\alpha_2$ integrin subunit expression as well. Additionally, we demonstrate that MiaPaCa-2 cells attach and proliferate strongly on vitronectin and fibronectin, and are very migratory on laminin.

Adhesion of pancreatic cancer cell lines to vitronectin yielded the most wide-ranging results, with MiaPaCa-2, Capan-1, AsPC-1, and Panc-1 exhibiting strong adhesion and proliferation (MiaPaCa-2 and AsPC-1), and CFPAC, FG, Colo-357, and BxPC-3 exhibiting weak adhesion and proliferation (CFPAC, FG, and BxPC-3). It is also interesting that three of the four cell lines demonstrating strong vitronectin adhesion, including Panc-1, MiaPaCa-2, and AsPC-1, also express wild-type SMAD4.

In summary, these studies using a broad panel of pancreatic cancer cell lines strongly implicate the $\alpha_2\beta_1$ integrin in the promotion of the malignant phenotype on type I collagen in pancreatic cancer, as defined by increased adhesion, proliferation, and migration. These results further identify the $\alpha_2\beta_1$ integrin as a potential therapeutic target in the treatment of this devastating disease. These studies also identify dramatic differences in integrin expression and ECM substrate preferences in pancreatic cancer cell lines expressing wild-type vs mutated SMAD4. Future studies will aim at the inhibition of pancreatic cancer growth, progression, and metastasis in our orthotopic mouse models using function-blocking monoclonals against $\alpha_2\beta_1$ integrin function.

ACKNOWLEDGEMENTS

We thank Drs Eva Engvall and Kristiina Vuori for helpful discussions and critical reading of the manuscript. This study was supported by a VA Merit grant from the Department of Veterans Affairs, National Institutes of Health Grant CA109949-01, American Cancer Society Grant RSG-05-037-01-CCE, and National Pancreas Foundation Grant.

REFERENCES

Armstrong T, Packham G, Murphy LB, Bateman AC, Conti JA, Fine DR, Johnson CD, Benyon RC, Iredale JP (2005) Type I collagen promotes the malignant phenotype of pancreatic ductal adenocarcinoma. Clin Cancer Res 10: 7427 – 7437

Bachem MG, Schunemann M, Ramadan M, Siech M, Beger H, Buck A, Zhou S, Schmid-Kotsas A, Adler G (2005) Pancreatic carcinoma cells induce fibrosis by stimulating proliferation and matrix synthesis of stellate cells. Gastroenterology 128: 907 – 921

Bardeesy N, DePinho RA (2002) Pancreatic cancer biology and genetics. Nat Rev Cancer 2: 897 – 909

Binkley CE, Zhang L, Greenson JK, Giordano TJ, Kucik R, Misek D, Hanash S, Logsdon CD, Simeone DM (2004) The molecular basis of pancreatic fibrosis: common stromal gene expression in chronic pancreatitis and pancreatic adenocarcinoma. Pancreas 29: 254 – 265

Chersah DA, Spiro RC (1987) Biosynthetic and functional properties of an Arg-Gly-Asp-directed receptor involved in human melanoma cell attachment to vitronectin, fibronogen, and von Willebrand factor. J Biol Chem 262: 17703 – 17711

Fabbri M, Castellani P, Gotwals PJ, Kotolianski V, Zardli L, Zocchi MR (1996) A functional monoclonal antibody recognizing the human alpha 1-integrin I-domain. Tissue Antigens 48: 47 – 51

Grzesiak JJ, Clroupon, P, Chalberg C, Burton DW, Silletti S, Moossa AR, Defos DJ, Bouvet M (2004) The extracellular matrix differentially regulates the expression of PTHrP and the PTH/PTHrP receptor in FG pancreatic cancer cells. Pancreas 29: 85 – 92

Grzesiak JJ, Davis GE, Kirchhofer D, Pierschbacher MD (1992) Regulation of alpha 2 beta 1 integrin expression as well. Additionally, we demonstrate that MiaPaCa-2 cells attach and proliferate strongly on vitronectin. tissue Antigens 254 – 259

Hoffman, in vitro. Endocrinology 146: 3567 – 3576

Iacobuzio-Donahue CA, Maitra A, Olsen M, Lowe AW, van Heek NT, Rosty C, Walter K, Sato N, Parker A, Ashfaq R, Jaffe E, Ryu B, Jones J, Eshleman JR, Yeo CJ, Cameron JL, Kern SE, Hruban RH, Brown PO, Goggins M (2003b) Exploration of global gene expression patterns in pancreatic adenocarcinoma using cDNA microarrays. Am J Pathol 162: 1151 – 1162

Kuehn R, Levi's Pl, Bloechle C, Niendorf A, Izbicki JR (1999) Angiogenesis, angiogenic growth factors, and cell adhesion molecules are upregulated in chronic pancreatic diseases: angiogenesis in chronic pancreatitis and in pancreatic cancer. Pancreas 18: 96 – 103

Linder S, Castanos-Velez E, von Rosen A, Biberfeld P (2001) Immunohistochemical expression of extracellular matrix proteins and adhesion molecules in pancreatic cancer. Hepatogastroenterology 48: 1321 – 1327

Loh M, Schmidt C, Ringel J, Kloth M, Muller P, Nizze H, Jesnowski R (2001) Transforming growth factor-beta induces desmoplasia in an experimental model of human pancreatic carcinoma. Cancer Res 61: 550 – 555

Loh M, Trautmann B, Grottler M, Peters S, Zauner I, Maier A, Kloppeg L, Liebe S, Kreuser ED (1996) Expression and function of receptors for extracellular matrix proteins in human ductal adenocarcinomas of the pancreas. Pancreas 12: 248 – 259

Loh M, Trautmann B, Grottler M, Peters S, Zauner I, Maiell B, Kloppeg L (1994) Human ductal adenocarcinomas of the pancreas express extracellular matrix proteins. Br J Cancer 69: 144 – 151

Menke A, Adler G (2002) TGFbeta-induced fibrogenesis of the pancreas. Int J Gastrointest Cancer 31: 41 – 46

Menke A, Philipp C, Vogelmann R, Seidel B, Lutz MP, Adler G, Wedlich D (2001) Down-regulation of E-cadherin gene expression by collagen type i and type III in pancreatic cancer cell lines. Cancer Res 61: 3508 – 3517

Miura K, Ujial J, Leabu M, Oravec T, Chakrabarti S, Morris VL, Chen BM (2005) Chemokine receptor CXCR4-beta integrin axis mediates tumorigenesis of osteosarcoma HOS cells. Biochem Cell Biol 83: 36 – 48

Miyamoto H, Murakami T, Tsuchida K, Sugino H, Miyake H, Tashiro S (2004) Tumor-stroma interaction of human pancreatic cancer: acquired resistance to anticancer drugs and proliferation regulation is dependent on extracellular matrix proteins. Pancreas 28: 38 – 44
Mollenhauer J, Roether I, Kern HF (1987) Distribution of extracellular matrix proteins in pancreatic ductal adenocarcinoma and its influence on tumor cell proliferation in vitro. Pancreas 2: 14–24

Monti P, Marchesi F, Reni M, Mercalli A, Sordi V, Zerbi A, Balzano G, Di Carlo V, Allavena P, Piemonti L. (2004) A comprehensive in vitro characterization of pancreatic ductal carcinoma cell line biological behavior and its correlation with the structural and genetic profile. Virchows Arch 445: 236–247

Nishimura S, Chung YS, Yashiro M, Inoue T, Sowa M. (1996) Role of alpha 2 beta 1- and alpha 3 beta 1-integrin in the periportal implantation of scirrhus gastric carcinoma. Br J Cancer 74: 1406–1412

Peng B, Fleming JB, Breslin T, Grau AM, Fojioka S, Abbruzzese JL, Evans DB, Ayers D, Wathen K, Wu T, Robertson KD, Chiao PJ. (2002) Suppression of tumorigenesis and induction of p15(ink4b) by Smad4/DP4 in human pancreatic cancer cells. Clin Cancer Res 8: 3628–3638

Ruoslahti E (1991) Integrins. J Clin Invest 87: 1–5

Ruoslahti E, Hayman EG, Pierschbacher M, Engvall E. (1982) Fibronectin: purification, immunological properties, and biological activities. Methods Enzymol 82(Part A): 803–831

Ruoslahti E, Noble NA, Kagani S, Border WA. (1994) Integrins. Kidney Int Suppl 44: S17–S22

Ruoslahti E, Pierschbacher MD. (1987) New perspectives in cell adhesion: RGD and integrins. Science 238: 491–497

Sawai H, Yamamoto M, Okada Y, Sato M, Akamo Y, Takeyama H, Manabe T. (2001) Alteration of integrins by interleukin-1alpha in human pancreatic cancer cells. Virchows Arch 444: 444–452

Sonnenberg A, Modderman PW, Hogervorst F. (1988) Laminin receptor on platelets is the integrin VLA-6. Nature 336: 487–489

Stein WD, Litman T, Fojo T, Bates SE. (2004) A Serial Analysis of Gene Expression (SAGE) database analysis of chemosensitivity: comparing solid tumors with cell lines and comparing solid tumors from different tissue origins. Cancer Res 64: 2805–2816

Tani T, Lumme A, Linnala A, Kivilaakso E, Kiviluoto T, Burgeson RE, Kangas L, Leivo I, Virtanen I. (1997) Pancreatic carcinomas deposit laminin-5, preferably adhere to laminin-5, and migrate on the newly deposited basement membrane. Am J Pathol 151: 1289–1302

Tempia-Caliera AA, Horvath LZ, Zimmermann A, Tihanyi TT, Korc M, Friess H, Buchler MW. (2002) Adhesion molecules in human pancreatic cancer. J Surg Oncol 79: 93–100

Vaquero EG, Edderkaoui M, Nam KJ, Gukovsky I, Pandol SJ, Gukovskaya AS. (2003) Extracellular matrix proteins protect pancreatic cancer cells from death via mitochondrial and nonmitochondrial pathways. Gastroenterology 125: 1188–1202

Vezederis MP, Meitner PA, Tibbetts LM, Doremus CM, Tzanakakis G, Calabresi P. (1990) Heterogeneity of potential for hematogenous metastasis in a human pancreatic carcinoma. J Surg Res 48: 51–55

Wayner EA, Carter WG. (1987) Identification of multiple cell adhesion receptors for collagen and fibronectin in human fibrosarcoma cells possessing unique alpha and common beta subunits. J Cell Biol 105: 1873–1884

Wayner EA, Carter WG, Piotrowicz RS, Kunicki TJ. (1988) The function of multiple extracellular matrix receptors in mediating cell adhesion to extracellular matrix: preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibit cell adhesion to fibronectin and react with platelet glycoproteins Ic-IIa. J Cell Biol 107: 1881–1891

Weinel RJ, Rosendahl A, Neumann K, Chaloupka B, Erb D, Rothmund M, Santos S. (1992) Expression and function of VLA-α1, -α3, -β1 and -α5 and -α6 6-integrin receptors in pancreatic carcinoma. Int J Cancer 52: 827–833