

# Type I Collagen Promotes the Malignant Phenotype of Pancreatic Ductal Adenocarcinoma

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## ABSTRACT

**Purpose:** The purpose of this study was to determine the role of functional interactions between pancreatic cancer cells and pancreatic stellate cells (PSCs) in the formation of the desmoplastic reaction (DR) in pancreatic cancer and to characterize the effect of type I collagen (the predominant component of the DR) on pancreatic cancer cell phenotype.

**Experimental Design:** PSCs and type I collagen were identified in sections of pancreatic cancer using immunohistochemistry, and their anatomic relationship was studied. Interactions among pancreatic cancer cell lines (MIA PaCa-2, Panc-1, and AsPC-1), primary cultures of human PSCs, and type I collagen were investigated in a series of tissue culture models.

**Results:** *In vivo*, the DR causes gross distortion of normal pancreas, bringing cancer cells into close contact with numerous PSCs and abundant type I collagen. In tissue culture models of pancreatic cancer, conditioned media from each cell line increased PSC [<sup>3</sup>H]thymidine incorporation up to 6.3-fold that of controls, and AsPC-1 cells also increased PSC collagen synthesis 1.3-fold. Type I collagen was observed to increase long-term survival of pancreatic cancer cells treated with 5-fluorouracil, by up to 62% in clonogenic assays. This was because type I collagen increased the proliferation of cancer cells (<sup>3</sup>H]thymidine incorporation was up to 2.8-fold that of cells cultured on tissue

culture plastic) and reduced apoptosis of AsPC-1 cells in response to 5-fluorouracil (by regulating mcl-1).

**Conclusions:** These experiments elucidate a mechanism by which the DR in pancreatic cancer may form and, via the collagen within it, promote the malignant phenotype of pancreatic cancer cells, suggesting significant detriment to the host.

## INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is characterized by an intense fibrotic reaction associated with the tumor, known as the desmoplastic reaction (DR; ref. 1). This manifests itself as bands of fibrous stroma surrounding malignant cells and results in a 3-fold increase in collagen compared with the normal pancreas (2–4). This is predominantly composed of collagen type I and, to a lesser degree, collagen type III, both of which are fibrillar collagens (3). Fibrillar collagens normally contribute to the structural integrity of epithelial organs by providing tensile strength to the interstitial matrix, which is separated from epithelial cells by basement membranes (5). In common with other epithelial malignancies, a key and prognostic event in pancreatic cancer is loss of basement membrane integrity and invasion of malignant cells into the interstitial matrix. This exposes malignant cells to interstitial collagens type I collagen of the DR (3, 6, 7).

Numerous myofibroblastic cells have been identified throughout the DR in pancreatic cancer and are often intimately related to malignant glands (8). Current evidence indicates that these are stellate cells, which are mesenchymal cells found in the liver and pancreas (9–11). They are characterized by their ability to transdifferentiate from a “quiescent” retinoid storing phenotype in the normal pancreas to an “activated” myofibroblastic phenotype in disease, indicated by  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) expression (10, 11). Myofibroblastic PSCs secrete extracellular matrix proteins, principally type I collagen, and express matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs), which regulate matrix turnover (10–13). It is emerging that PSCs play a central role in chronic pancreatitis, in which the pathological accumulation of fibrillar collagen is central to the disease process (14, 15). Analysis of collagen in chronic pancreatitis and PDAC demonstrates marked homology in composition between the two, implicating a common cellular source (4). Although extracellular matrix is known to regulate phenotype, proliferation, and survival of multiple cell types, very little is known about the functional interactions among malignant pancreatic cells, PSCs, and type I collagen and their effects on the malignant phenotype of pancreatic cancer (3, 16, 17).

In this study, we present novel evidence for interplay between pancreatic cancer cells and PSCs. We show that pancreatic cancer cells promote proliferation and collagen synthesis by PSCs. We also demonstrate that the major product of acti-

Received 12/30/03; revised 5/17/04; accepted 7/28/04.

**Grant support:** A Wellcome Trust Research Training Fellowship and a Royal College of Surgeons of Edinburgh pump-priming grant awarded to T. Armstrong. G. Packham is supported by Cancer Research UK. J. Iredale and R. Benyon are members of a Medical Research Council COGG, and J. Iredale is a Medical Research Council Senior Clinical Fellow.

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vated PSCs, type I collagen, confers a survival advantage to the pancreatic cancer cells by regulating proliferation and apoptosis.

## MATERIALS AND METHODS

**Cell Culture.** Pancreatic cancer cell lines MIA PaCa-2 and Panc-1 (derived from primary tumors) were purchased from European Collection of Cell Cultures (Wiltshire, United Kingdom), and AsPC-1 (derived from metastasis) was purchased from American Type Culture Collection (Manassas, VA). They were cultured at 37°C with 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with L-glutamine, penicillin, streptomycin, and gentamicin and 10% (v/v) heat-inactivated fetal calf serum (FCS; Invitrogen). All cells were passaged with 0.25% (v/v) trypsin (Biomedica) and used for a maximum of 20 passages. Cell viability was routinely checked after passage by trypan blue exclusion and was consistently >95%.

**Isolation of Pancreatic Stellate Cells.** PSCs were isolated from normal pancreas taken from specimens removed from patients undergoing surgical resection for malignancy or from tissue removed in the course of surgical treatment of chronic pancreatitis according to a method originally described by Apte *et al.* (11) with minor modifications (12). Briefly, the pancreas was digested using a solution of bacterial Pronase, collagenase, and DNase. After centrifugation, the cell pellet was resuspended in Optiprep (Sigma, Poole, Dorset, United Kingdom) at a concentration of 12% (v/v) in Hanks' balanced salt solution (HBSS), with HBSS layered on top before density centrifugation. Cells were harvested from the density interface and cultured in DMEM supplemented with 16% FCS and antibiotics. All experiments were performed using three or more separate isolates of human PSCs, between passages 2 and 5.

**Immunohistochemistry.** Six tissue blocks were identified from patients who had recently undergone pancreatic resection for PDAC and three sections of normal pancreas were obtained after Whipple's operation for duodenal neoplasia. Paraffin-fixed sections (5 μm) were immunostained with antibodies to αSMA (clone 1A4; Sigma) and type I collagen (clone COL-1; Abcam, Cambridge, United Kingdom) by using standard laboratory methods (12).

**Conditioned Media.** Conditioned supernatant was obtained by culturing subconfluent layers of MIA PaCa-2, Panc-1, and AsPC-1 cells in DMEM containing 0.5% FCS or 0.01% bovine serum albumin (BSA; Sigma) for 24 hours. The supernatant was centrifuged (500 × *g* for 5 minutes) and filter sterilized (0.2 μm) and then diluted in equal quantities with fresh 0.5% FCS or 0.01% BSA (v/v) DMEM before use in experiments. In all experiments using conditioned media, cells were initially cultured in normal medium for 24 hours; normal medium was removed, and the cells were washed twice with HBSS before addition of conditioned medium or appropriate control.

**Measurement of [<sup>3</sup>H]Thymidine Incorporation.** The effect of supernatant transfer and collagen on proliferation of cancer cells and PSCs was measured using [<sup>3</sup>H] thymidine incorporation. Experiments were conducted in triplicate in 24-well plates. Before the addition of 1 μCi of [*methyl*-<sup>3</sup>H]thymidine (Amersham Biosciences, Buckinghamshire, United King-

dom), cells were incubated with medium containing 0.5% FCS for 6 hours. Cells were then cultured for an additional 16 hours (with conditioned medium or on collagen) in the presence of [<sup>3</sup>H]thymidine, washed with HBSS, and fixed in ice-cold methanol. The fixed cells were solubilized in 0.25 mol/L sodium hydroxide/0.2% (w/v) SDS (Sigma) and neutralized with 5 mol/L hydrochloric acid, the lysates were added to OptiPhase HiSafe 3 scintillation fluid (Wallac, Turku, Finland), and scintillation was counted using a MicroBeta 1450 Scintillation Counter (Elkay). When this technique was used to compare proliferation of cancer cells on tissue culture plastic (TCP) *versus* collagen, potential variation in adhesion to each matrix was controlled by quantifying total DNA.

**Quantification of DNA.** Total DNA was quantified in assays, where indicated, to control for changes in cell proliferation or adhesion in the case of experiments on collagen. DNA was quantified using PicoGreen Reagent (Molecular Probes, Eugene, OR), either directly from the cell monolayer or from parallel duplicate wells in experiments using [<sup>3</sup>H] thymidine/hydroxyproline, by methods described previously (18).

**Quantification of Stellate Cell Procollagen I RNA by TaqMan Quantitative Real-Time Polymerase Chain Reaction.** RNA was extracted using Qiagen RNeasy RNA extraction kit. RNA integrity was routinely verified by demonstrating undegraded 28S and 18S ribosomal bands in RNA, separated on a 1% agarose gel. Subsequently, cDNA was prepared from 1 μg of RNA using a proprietary kit (Reverse Transcription System; Promega, Madison, WI), and equal volumes of first-strand cDNA were subjected to TaqMan analysis. The sequences of primers (used at 0.5 μmol/L) and probes (used at 1 μmol/L) used for procollagen I in the TaqMan were as follows: sense, 5'-caagaggaaggccaagtgcagg-3'; antisense, 5'-cggtgtcgcagcagcat-3' (Oswel Laboratories); and probe, 5'-cctcaggtaccatgaccgagcgtgtggaacc-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequences were as follows: sense, 5'-gaaggtgaaggtcgagtc-3'; antisense, 5'-gaagatggtgatggatttc-3'; and probe, 5'-caagctcccgtctcagcc-3' (Applied Biosystems, Foster City, CA). The conditions for the reaction (after initial hold at 50°C for 2 minutes and 95°C for 10 minutes) were 95°C for 15 seconds (denaturing) then 60°C for 60 seconds (annealing). All reactions were performed in triplicate, and the results obtained for procollagen I were normalized to those obtained in parallel TaqMan quantification of mRNA for the housekeeping gene GAPDH according to Pfaffl (19).

**Quantification of Stellate Cell Collagen Synthesis by [<sup>3</sup>H]Hydroxyproline Incorporation.** Stellate cells were grown to subconfluent monolayers in duplicate wells of 12-well plates, at which point media were replaced with cancer cell conditioned media [made with DMEM containing 0.01% (w/v) BSA by the method above], supplemented with 25 μg/mL ascorbic acid. After 24 hours of culture, 1 μCi of L-[2,3,4,5-<sup>3</sup>H]hydroxyproline (Amersham Biosciences) was added to the cells, allowing incorporation into newly synthesized collagen. After an additional 24 hours of incubation, cells were processed using the method described previously, and results are expressed as cpm per microgram of cellular DNA (measured using PicoGreen; ref. 20).

**Transforming Growth Factor  $\beta$ 1 Enzyme-Linked Immunosorbent Assay.** Total TGF- $\beta$ 1 secreted in cancer cell conditioned media was measured by activating latent TGF- $\beta$ 1 with hydrochloric acid and completing the protocol of a commercial enzyme-linked immunosorbent assay (ELISA) exactly according to the manufacturer's instructions (Bio-Rad, Hercules, CA). TGF- $\beta$ 1 concentration was derived from a standard curve generated with recombinant TGF- $\beta$ 1 (as supplied).

**MMP-2 Activity Assay and TIMP-1 ELISA.** A total of  $2.5 \times 10^4$  PSCs or cancer cells were cultured in 12-well plates for 24 hours in normal medium. After 24 hours, the medium was replaced with 500  $\mu$ L of 0.5% FCS (v/v) DMEM and incubated for an additional 24 hours. MMP-2 and TIMP-1 were subsequently measured in the conditioned medium using a proprietary MMP-2 Biotrak Activity Assay System or TIMP-1 Biotrak ELISA System (Amersham Biosciences) exactly following the manufacturer's instructions. The MMP-2 bound to anti-MMP-2 antibody-coated 96-well plates. The captured MMP-2 was activated using *p*-aminophenylmercuric acetate. The activated MMP-2, in turn, catalyzed activation of a urokinase detection enzyme that degraded a colorimetric substrate, the absorbance of which is detected at 405 nm. Results were normalized to total DNA measured using PicoGreen to account for different rates of cell proliferation.

**Culture of Cells on Collagen.** TCP (Greiner, Frickenhausen, Germany) was coated with 15  $\mu$ g/cm<sup>2</sup> type I collagen (Sigma, Gillingham, United Kingdom) dissolved in 0.1 mol/L acetic acid. The collagen was left to adhere overnight at 4°C, the acid was washed off with HBSS, and the surface was blocked with 0.1% (w/v) BSA/DMEM for 1 hour at room temperature, which was removed by washing with HBSS. Control TCP wells were also blocked with BSA. In all experiments using collagen, the cancer cells were cultured in normal medium supplemented with 0.1 mmol/L Mn<sup>2+</sup> and allowed to adhere to the matrix for 24 hours. These measures were taken to prevent nonspecific adhesion and promote integrin binding in these experiments (21).

**Clonogenic Assay.** Cells were cultured in a 25-cm<sup>2</sup> flask on TCP or type I collagen in 0.5% FCS or 0.5% FCS containing 5-fluorouracil (5-FU) for 24 hours. Cells from each treated and untreated pair were trypsinized and seeded in quadruplicate at 200 cells per well and left for 8 to 14 days until colonies had formed. Colonies were visualized with Giemsa stain (Sigma) and counted. The number of colonies (>16 cells) formed by cells exposed to 5-FU was divided by the number of colonies in the untreated pair, giving an index of viability derived from each substratum.

**Fluorescence-Activated Cell-Sorting Analysis.** Pancreatic cancer cells ( $2.4 \times 10^6$ ) were subjected to a 1-hour pulse of 25 mmol/L bromodeoxyuridine (BrdUrd; Becton Dickinson, San Jose, CA) while being cultured on TCP or type I collagen in 75-cm<sup>2</sup> cell culture flasks. Cells were harvested and fixed at this stage (1 hour time point), or the BrdUrd was removed and replaced with fresh culture medium, and cells were harvested at increasing time intervals. Cells were harvested by trypsinization, fixed in cold EtOH, and then prepared for fluorescence-activated cell-sorting (FACS) analysis following the protocol

published on the Cancer Research United Kingdom website.<sup>3</sup> Briefly, DNA was denatured using 2 mol/L hydrochloric acid and BrdUrd labeled primarily with anti-BrdUrd antibodies and then with fluorescein isothiocyanate (FITC)-conjugated F(ab')<sub>2</sub> secondary antibodies (both from DAKO, United Kingdom). The cells were counterstained with 5 mg/mL propidium iodide (PI) in PBS containing 2 mg/mL RNase A (both from Sigma, Gillingham, United Kingdom). FACS analysis was performed using standard protocols on a Becton Dickinson flow cytometer, and 10,000 events were routinely acquired. Doublets were excluded from further analysis by gating. The remaining events were plotted according to DNA content (PI staining, channel FL-2) and FITC labeling (channel FL-1 log scale), making it possible to identify cells in G<sub>1</sub>, S, and G<sub>2</sub>M phases (Fig. 9B).

**Western Analysis.** A total of  $8 \times 10^5$  cancer cells were seeded in 25-cm<sup>2</sup> culture flasks on TCP or type I collagen prepared as described above. After 24 hours, the medium was replaced with 0 to 100  $\mu$ g/mL 5-FU (F. H. Faulding & Co. Ltd.) and incubated for an additional 72 hours. Stellate cells were cultured under standard conditions. Western analysis was performed according to Brimmell *et al.* (22) using the following antibodies: poly(ADP-ribose) polymerase [PARP (clone C2-10; R&D Systems, Minneapolis, MN)],  $\alpha$ SMA (clone 1A4; Sigma), bak (clone N20; Santa Cruz Biotechnology, Santa Cruz, CA), bcl<sub>xL</sub> (clone AF800; R&D Systems), bax (clone N20; Santa Cruz Biotechnology), mcl-1 (clone S19; Santa Cruz Biotechnology), and  $\beta$ -actin (clone AC-15; Sigma). These antibodies were applied in 5% milk diluted in PBS, Tris-buffered saline, or 0.05% Tween (Sigma) v/v tris buffered saline, followed by appropriate horseradish peroxidase-conjugated secondary antibody (all from Amersham Biosciences; ref. 22). Reactive bands were identified by chemiluminescent substrate (Super Signal; Pierce, Rockford, IL), imaged, and analyzed using a Fluor S Multimager and software (Bio-Rad).

**Cell Counting Experiments.** Cells were prepared in 25-cm<sup>2</sup> flasks using an identical method described for Western analysis and treated with 5-FU (25  $\mu$ g/mL). Detached and adherent cells (collected after trypsinization) were suspended in equal volumes of PBS and counted using a hemocytometer. The mean cell count from four grids was determined in triplicate. Detached and adherent cells were stained with acridine orange (1  $\mu$ g/mL; Sigma), allowing identification of the morphologic changes of apoptosis, and subjected to Western analysis for PARP.

**Statistical Analysis.** Statistical differences between groups in these assays were calculated using a Student's two-tailed *t* test. Significance was defined as  $P < 0.05$ .

## RESULTS

**Histologic Changes in Type I Collagen Expression and PSC Distribution in Pancreatic Cancer.** The DR in PDAC has previously been reported to contain an abundance of type I collagen and numerous  $\alpha$ SMA-positive PSCs (2, 3, 8). This was confirmed by immunostaining sections from six randomly se-

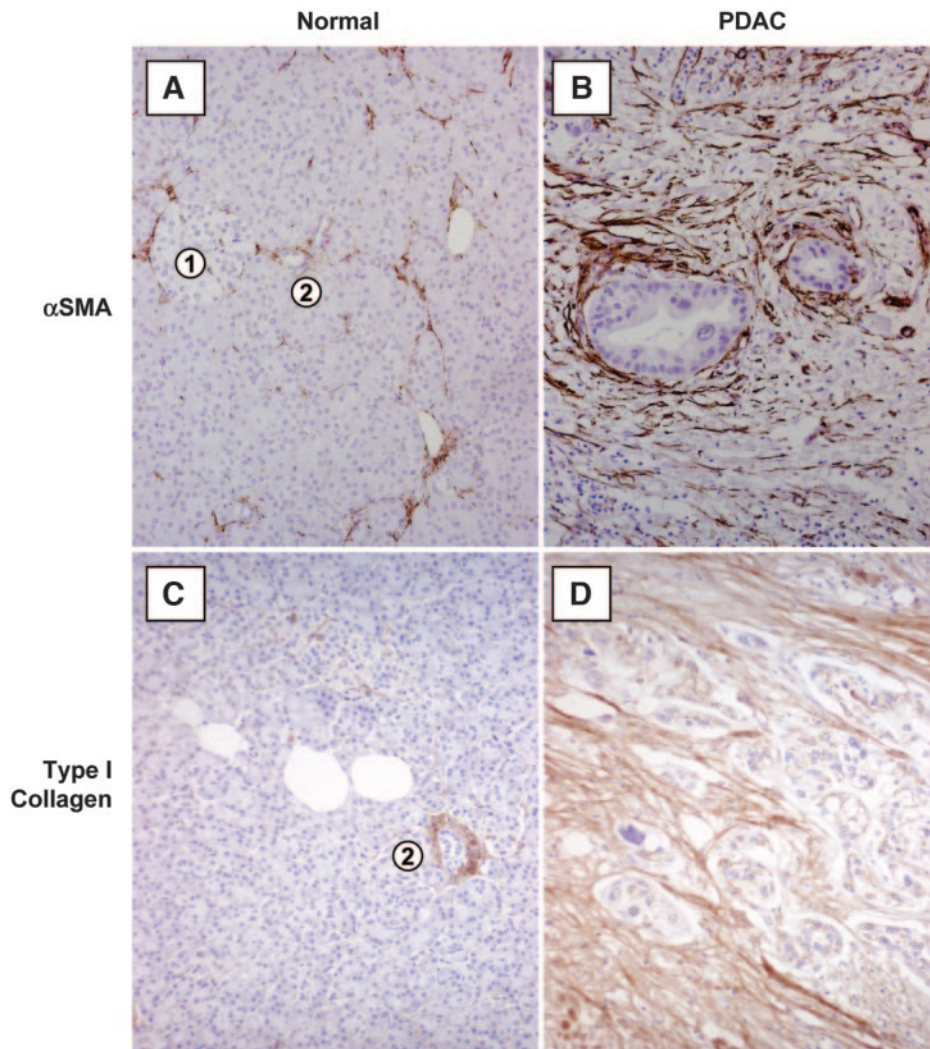
<sup>3</sup> www.icnet.uk.

lected patients who had undergone pancreaticoduodenectomy for pancreatic cancer at Southampton General Hospital. In the normal pancreas (removed for duodenal neoplasia), occasional  $\alpha$ SMA-expressing stellate cells were identified in interlobular septa and the connective tissue supporting pancreatic ductules (Fig. 1A). In the neoplastic tissue, however, there was a marked increase in stellate cell number in the fibrotic bands, particularly around malignant glands (Fig. 1B). Similarly, in the normal pancreas, type I collagen was confined to the connective tissue surrounding larger pancreatic ducts (Fig. 1C), in contrast to large tracts that surround the malignant epithelium in PDAC (Fig. 1D). These anatomic studies illustrate the close spatial relationship that exists among malignant epithelial cells, PSCs, and type I collagen in PDAC. Secondly, they demonstrate the dramatic increase in the proportion of stromal elements of PDAC compared with the normal pancreas.

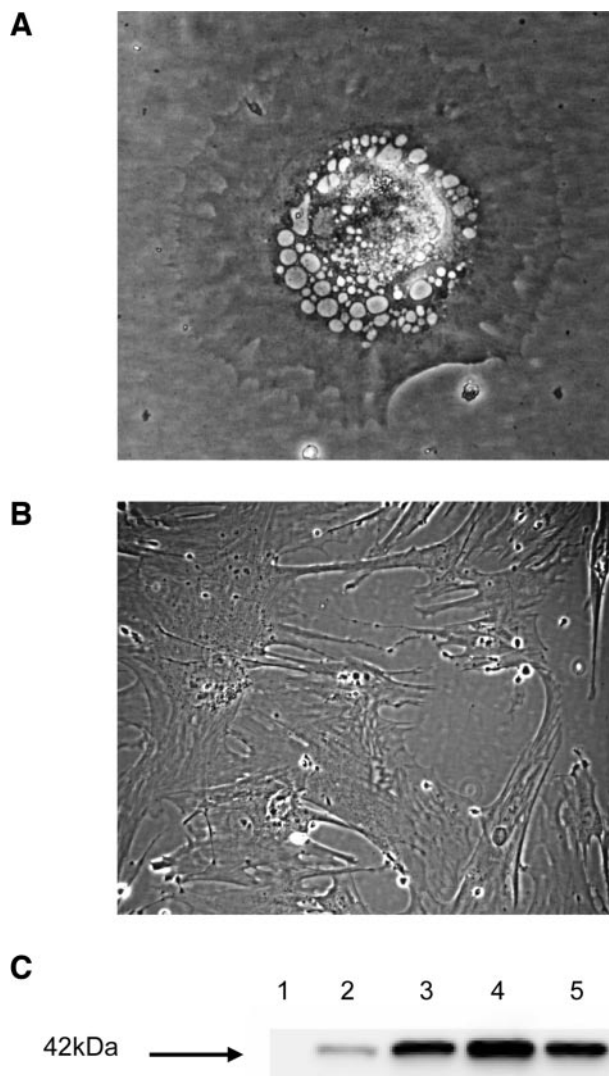
**Effect of Cancer Cell Conditioned Medium on Proliferation and Collagen Secretion in PSCs.** It was hypothesized that the pancreatic cancer cells may stimulate PSC growth and collagen secretion in PDAC, thus promoting the develop-

ment of the DR. To determine whether this was the case, a series of experiments were conducted *in vitro* using tissue culture models of PDAC. The culture models relied on three pancreatic cancer cell lines and PSCs isolated from human pancreas. Typically,  $0.25$  to  $0.75 \times 10^6$  PSCs were isolated and became adherent and activated over a period of 14 days. Initially, the cells had morphologic features of quiescent PSCs, containing lipid/retinoid droplets in the cytoplasm (Fig. 2A). These features were lost with transformation to the myofibroblastic phenotype (Fig. 2B). The myofibroblastic phenotype was subsequently verified by Western analysis for  $\alpha$ SMA (Fig. 2C).

Pancreatic cancer cell conditioned supernatant was transferred to primary cultures of PSCs, and its effect on proliferation was measured by [ $^3$ H]thymidine incorporation. Supernatant from MIA PaCa-2, Panc-1, and AsPC-1 cells significantly increased stellate cell [ $^3$ H]thymidine incorporation by up to 6-fold compared with cells cultured in 0.5% FCS alone (Fig. 3A). Serial dilution of the conditioned supernatant demonstrated that the effect on [ $^3$ H]thymidine incorporation was concentration dependent (Fig. 3B).



*Fig. 1* Immunohistochemical analysis of normal pancreas and pancreatic cancer specimens for  $\alpha$ SMA and type I collagen (magnification,  $\times 20$ ). A and B, normal pancreas/pancreatic cancer stained for  $\alpha$ SMA. C and D, normal pancreas/pancreatic cancer stained for type I collagen. 1, islet of Langerhans; 2, pancreatic ductule.

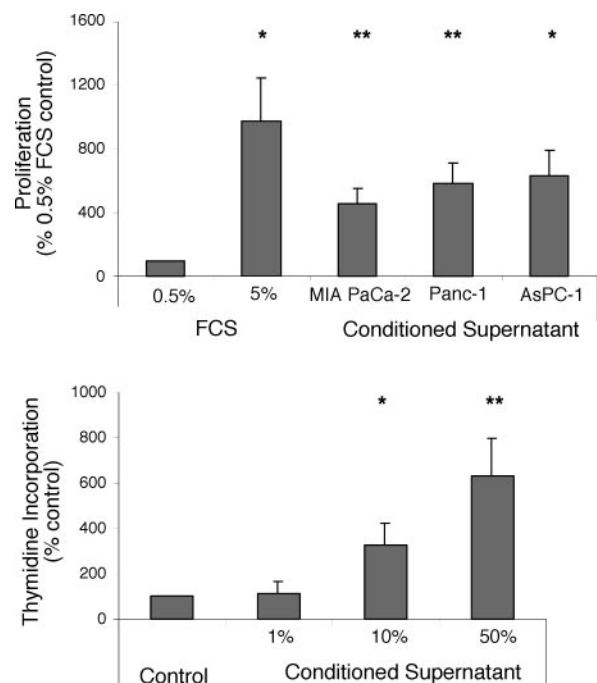


**Fig. 2** Isolated human PSCs. **A**, photomicrograph of freshly isolated human PSCs after 4 days of culture on TCP, demonstrating numerous cytoplasmic lipid droplets (magnification,  $\times 400$ ). **B**, photomicrograph of isolated human PSCs cultured on TCP for 21 days (magnification,  $\times 200$ ). **C**, Western analysis for  $\alpha$ SMA expression in three separate primary cultures of human PSCs after two passages, demonstrating the myofibroblastic phenotype of the activated PSCs. *Lane 1*, MIA PaCa-2 cells (negative control); *Lane 2*, human hepatic stellate cell line (positive control); *Lanes 3–5*, PSCs.

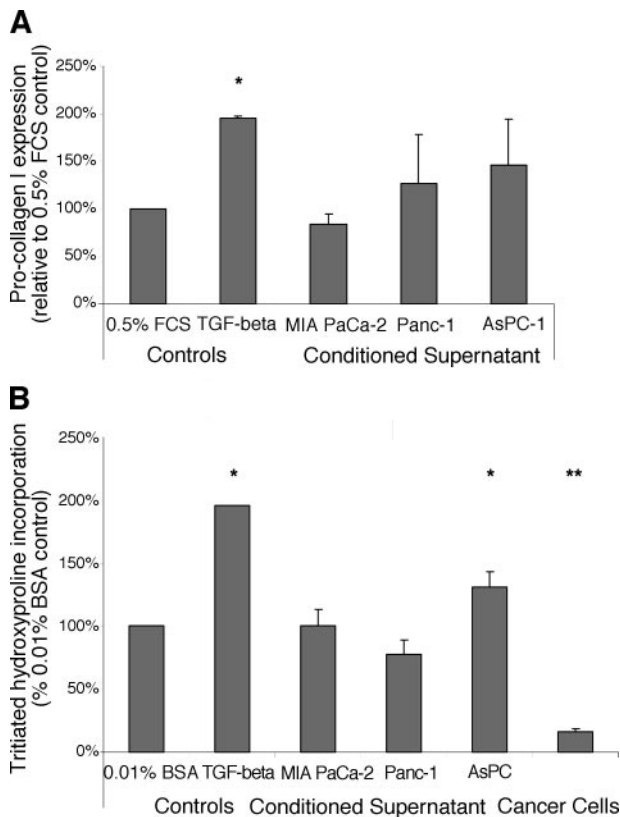
PSCs are known to secrete collagen and type I collagen in particular (23). Experiments were therefore undertaken to determine whether conditioned supernatant from pancreatic cancer cells also regulated type I collagen synthesis. PSC procollagen I mRNA was measured using TaqMan reverse transcription-polymerase chain reaction. Procollagen I mRNA was readily detectable in the PSCs with a mean number of PCR cycles to fluorescent threshold of 20.7, compared with 26.0 for GAPDH in cells cultured in 0.5% FCS. After 24 hours of incubation with Panc-1 and AsPC-1 conditioned medium, there appeared to be a modest, nonsignificant increase in procollagen I mRNA expression by PSCs (Fig. 4A). The effect of cancer cell conditioned

medium on collagen synthesis was also assayed at protein level by measuring tritiated hydroxyproline incorporation (24). Although not specific to type I collagen, the results obtained using this technique were important because it accounted for the extensive posttranscriptional regulation that collagen synthesis is subject to (24). These experiments demonstrated that AsPC-1 conditioned medium increased PSC collagen synthesis compared with controls (Fig. 4B), whereas Mia PaCa-2 and Panc-1 conditioned media did not appear to stimulate PSC collagen synthesis. Recombinant TGF- $\beta$ 1 (used as a positive control) consistently increased collagen expression at RNA and protein level by PSCs. TGF- $\beta$ 1 was also detected in conditioned supernatants of all three cell lines by ELISA, at significantly greater levels than controls [0.5% FCS (v/v) DMEM; Fig. 5]. Collagen synthesis by cancer cells was measured using the tritiated hydroxyproline assay and found to be approximately 5-fold less than that by PSCs (Fig. 4B).

MMPs and TIMPs regulate turnover of secreted collagen, and therefore MMP-2 and TIMP-1 were measured in the conditioned media of pancreatic cancer cells and PSCs (Fig. 6). This demonstrated that PSCs express MMP-2 at between 7.2-fold and 28.4-fold that of the pancreatic cancer cell lines tested (Fig. 6A). Furthermore, PSCs expressed TIMP-1 at between 8.1-



**Fig. 3** Effect of supernatant transfer from pancreatic cancer cells to PSCs. **A**, PSCs ( $1.5 \times 10^4$  cells) were cultured with 0.5% FCS, 5% FCS (positive control), and cancer cell conditioned medium from each cell line for 16 hours, during which time [ $^3$ H]thymidine incorporation was measured. *Bars* represent the mean  $\pm$  SE of six independent experiments. **B**, the effect of conditioned media from AsPC-1 cells, serially diluted with fresh 0.5% FCS on PSC [ $^3$ H]thymidine incorporation over 16 hours. *Bars* represent the mean  $\pm$  SE of three independent experiments (similar results were obtained using conditioned media from MIA PaCa-2 and Panc-1 cells). Data are presented as a percentage of control (0.5% FCS), which was given an arbitrary value of 100%. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (with respect to 0.5% FCS control).



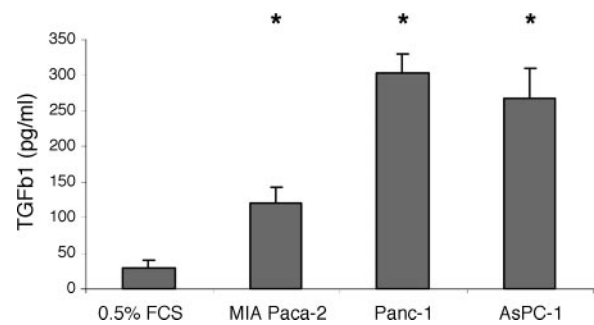
**Fig. 4** Effect of cancer cell supernatant on PSC collagen synthesis. TGF- $\beta$ 1 is known to induce PSC collagen synthesis and was used as a positive control (10  $\mu$ g/mL). **A**. PSCs were incubated with 0.5% FCS, TGF- $\beta$ 1, or MIA PaCa-2/Panc-1/AsPC-1 conditioned media for 24 hours. RNA was extracted, and procollagen I/GAPDH mRNA expression was measured by TaqMan reverse transcription-polymerase chain reaction. Cycle threshold ( $C_T$ ) for procollagen I was normalized to  $C_T$  for GAPDH, and the data are presented as a percentage of the results obtained with controls (0.5% FCS), which was given an arbitrary value of 100%. Bars represent the mean of three independent experiments  $\pm$  SE. **B**. Collagen synthesis by PSCs cultured under the same experimental conditions was also measured by [ $^3$ H]hydroxyproline incorporation (allowing measurement of collagen at the protein level). The results were normalized to total DNA to account for any changes in PSC proliferation. Collagen synthesis by MIA PaCa-2, Panc-1, and AsPC-1 cells was also measured, and the mean of these results (normalized to PSCs) are included on the chart. Data are presented as a percentage of control, which was given an arbitrary value of 100%. Bars represent the mean of a minimum of three independent experiments  $\pm$  SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (with respect to control).

and 16.6-fold that of the cell lines. PSCs would therefore appear to be the principle source of MMP-2 and TIMP-1 in pancreatic cancer. TIMP-1 secretion by PSCs was approximately 200-fold that of MMP-2.

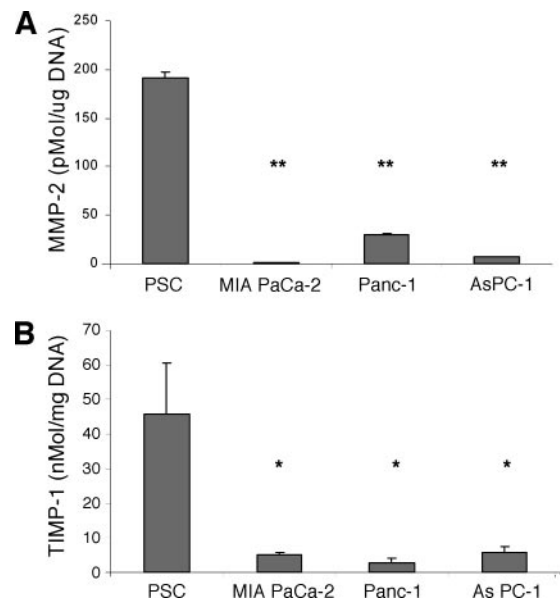
Together, these experiments indicate that PSCs are likely to be the major cellular source of collagen in the DR. Secondly, via soluble factors, pancreatic cancer cells have the potential to increase collagen secretion by PSCs, principally by promoting their proliferation but also by regulating aspects of PSC collagen synthesis, possibly via TGF- $\beta$ 1. PSCs would also appear to be the principle source of MMPs and TIMPs in pancreatic cancer,

and relatively high levels of TIMP-1 would promote the accumulation of the extracellular matrix in pancreatic cancer.

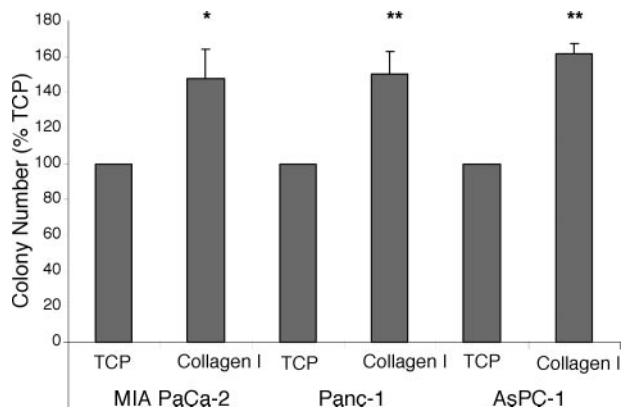
**Effect of Type I Collagen on Chemosensitivity of Pancreatic Cancer Cells.** The potential effect of the abundant type I collagen on the phenotype of pancreatic cancer cells was initially tested by determining its effect on chemosensitivity using clonogenic assays. Panc-1 and AsPC-1 cells were cultured on TCP or purified type I collagen and exposed to 5-FU. This was subsequently removed, and the ability of cells to form colonies was measured. Type I collagen did not alter subsequent colony-forming efficiency by untreated controls. Treatment with 5-FU led to a reduced colony forming efficiency ( $32.0 \pm 7.6\%$



**Fig. 5** TGF- $\beta$ 1 concentration in cancer cell conditioned media was measured using a commercial ELISA. Bars represent the mean of three independent experiments  $\pm$  SE. \*,  $P < 0.05$  with respect to TGF- $\beta$ 1 contained in 0.5% FCS (v/v)/DMEM.



**Fig. 6** MMP-2 and TIMP-1 expression by PSCs and pancreatic cancer cells. Cells ( $2.5 \times 10^4$ ) were seeded in 12-well plates and cultured in 0.5% FCS (v/v)/DMEM. After 24 hours, the medium was removed, and secreted MMP-2 (**A**) and TIMP-1 (**B**) were measured using the MMP-2 Biotrak Activity Assay and TIMP-1 ELISA, respectively. Culture medium alone (0.5% FCS (v/v)/DMEM) contained 1.4 pmol/L MMP-2 and 1.3 nmol/L TIMP-1. Bars represent the mean of three independent experiments  $\pm$  SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (with respect to results obtained from PSCs).



**Fig. 7** The effect of type I collagen on the long-term clonogenic potential of pancreatic cancer cells in clonogenic assays. MIA PaCa-2, Panc-1, and AsPC-1 cells were incubated in 0.5% FCS alone or 0.5% FCS containing 5-FU (3  $\mu\text{g}/\text{mL}$  for Mia PaCa-2, 7  $\mu\text{g}/\text{mL}$  for Panc-1, and 5  $\mu\text{g}/\text{mL}$  for AsPC-1) for 24 hours while being cultured on TCP or type I collagen. Cells were then trypsinized and seeded in equal numbers onto TCP in 24-well plates in normal medium. After 8 to 14 days, the number of colonies of >16 cells were counted, and an index of cell viability was determined by dividing the number of colonies formed by 5-FU-treated cells with controls. The results obtained were normalized to those on TCP, which was given a value of 100%. The bars represent a mean of five independent experiments  $\pm$  SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (with respect to TCP).

for MIA PaCa-2,  $21.1 \pm 4.1\%$  for Panc-1, and  $28.7 \pm 1.5\%$  for AsPC-1), but MIA PaCa-2, Panc-1, and AsPC-1 cells that had been cultured on type I collagen during treatment subsequently formed approximately 50% more colonies than those cultured on TCP (Fig. 7). This indicated that type I collagen must provide either growth-promoting or antiapoptotic stimuli to the cancer cells, providing some protection from the cytotoxic effect of 5-FU. The potential contributions of proliferation and apoptosis were therefore investigated.

**Effect of Type I Collagen on Proliferation of Pancreatic Cancer Cells.** Pancreatic cancer cells cultured on purified type I collagen assumed a different morphology. Panc-1 and AsPC-1 cells readily attached to type I collagen and tended to become flattened, spreading out and growing individually, compared with small clusters of cells that were observed on plastic (Fig. 8). In contrast, MIA PaCa-2 cells formed clusters of small round cells.

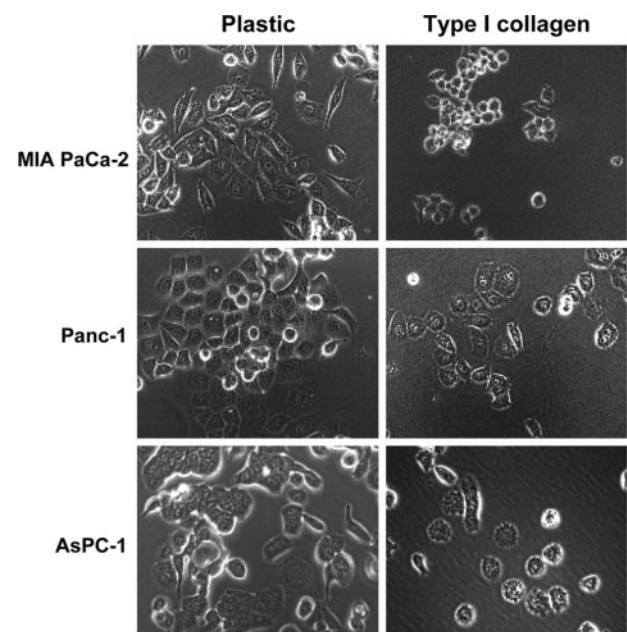
Type I collagen also consistently increased [ $^3\text{H}$ ]thymidine incorporation in all pancreatic cancer cell lines (Fig. 9A). The proliferative effect of type I collagen was studied by flow cytometric analysis of cells stained with FITC-BrdUrd/PI. This indicated that type I collagen did not alter the proportion of cells in  $G_0$ - $G_1$ , S, or  $G_2$ -M phases (data not shown). Cell cycle kinetics was therefore studied by exposing cells to a pulse of BrdUrd, which was incorporated by cells actively replicating DNA (S phase). The cells were harvested between 1 and 21 hours after the pulse, and BrdUrd-positive cells were detected by FITC-labeled antibodies. The BrdUrd-positive cells were gated from a dot plot (Fig. 9B), and histograms were generated according to DNA content of the cells (which, during S phase, increased from 2n in  $G_1$  to 4n in  $G_2$  before dropping to 2n after

mitosis; Fig. 9C). It was observed that a larger proportion of cells cultured on type I collagen transited the cell cycle and accumulated in  $G_1$  phase after 14 to 21 hours than those cultured on plastic, implying a more rapid transition through S phase and/or  $G_2$ -M.

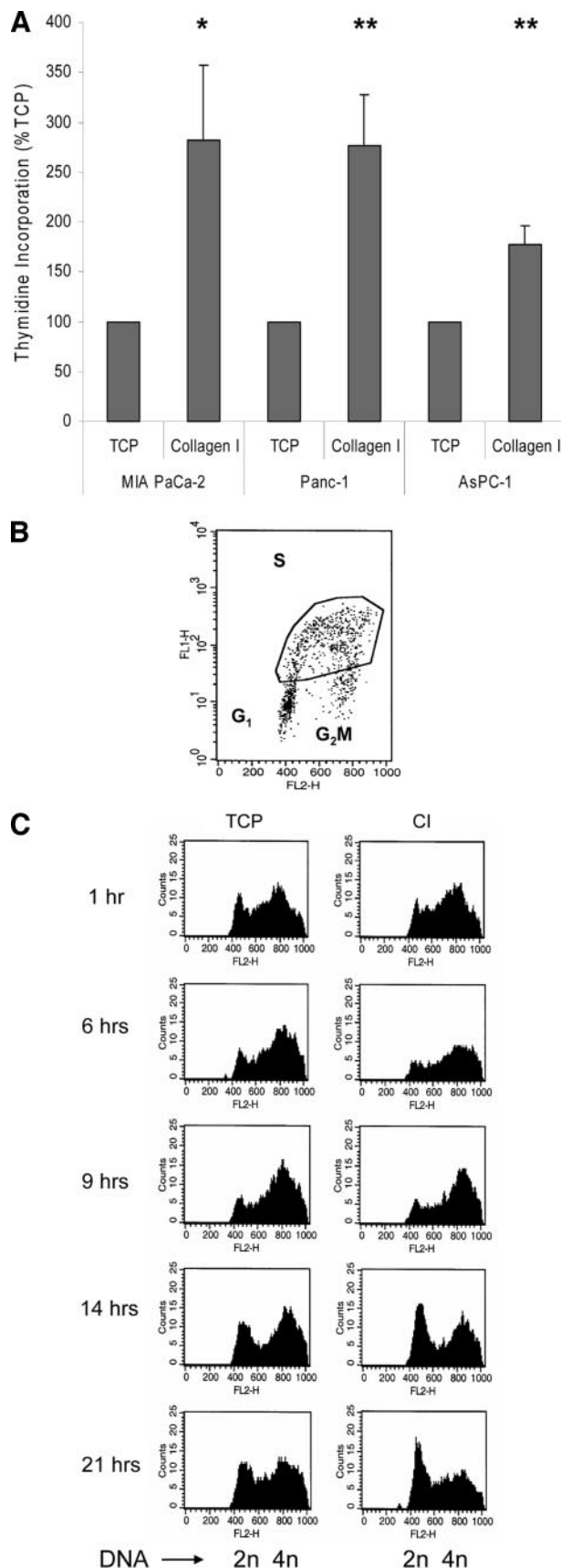
These experiments indicated that type I collagen supported proliferation of MIA PaCa-2, Panc-1, and AsPC-1 cells compared with TCP.

**Effect of Type I Collagen on Pancreatic Cancer Cell Apoptosis.** Regulation of proliferation is often accompanied by regulation of apoptosis (25). MIA PaCa-2, Panc-1, and AsPC-1 cells treated with 5-FU all contained the cleaved form of PARP (a substrate for caspases, activated during apoptosis) and also exhibited nuclear condensation/fragmentation, indicating that 5-FU induced apoptosis (for examples, see Fig. 10). A relative reduction in PARP cleavage was observed in AsPC-1 cells cultured on type I collagen compared with TCP (Fig. 10A), but there was no evidence of this effect in MIA PaCa-2 or Panc-1 cells (data not shown). The suppressive effect of type I collagen on apoptosis in AsPC-1 cells was confirmed by counting the proportion of detached and adherent cells after treatment with 5-FU on TCP and type I collagen (Fig. 10B). Type I collagen reduced the proportion of detached cells by 14.0% compared with plastic ( $P < 0.05$ ). The detached cells were all apoptotic as judged by acridine orange staining and Western analysis for PARP, which was exclusively of the cleaved form (Fig. 10C and D). In contrast, the adherent cells had normal nuclear morphology and expressed intact PARP (Fig. 10C and D).

A series of Bcl-2-related proteins were studied by Western analysis to investigate the mechanism underlying regulation of apoptosis in AsPC-1 cells by type I collagen (Fig. 10E). Anti-



**Fig. 8** Photomicrographs demonstrating morphological appearances of the cancer cells cultured on type I collagen for 24 hours (magnification,  $\times 100$ ).



apoptotic Mcl-1 was expressed at a higher level in cells cultured on type I collagen (compared with TCP), but cell culture matrix did not appear to regulate Bak, Bax, or Bcl<sub>XL</sub> expression. These data provide a potential mechanism by which type I collagen confers resistance to apoptosis in AsPC-1 cells.

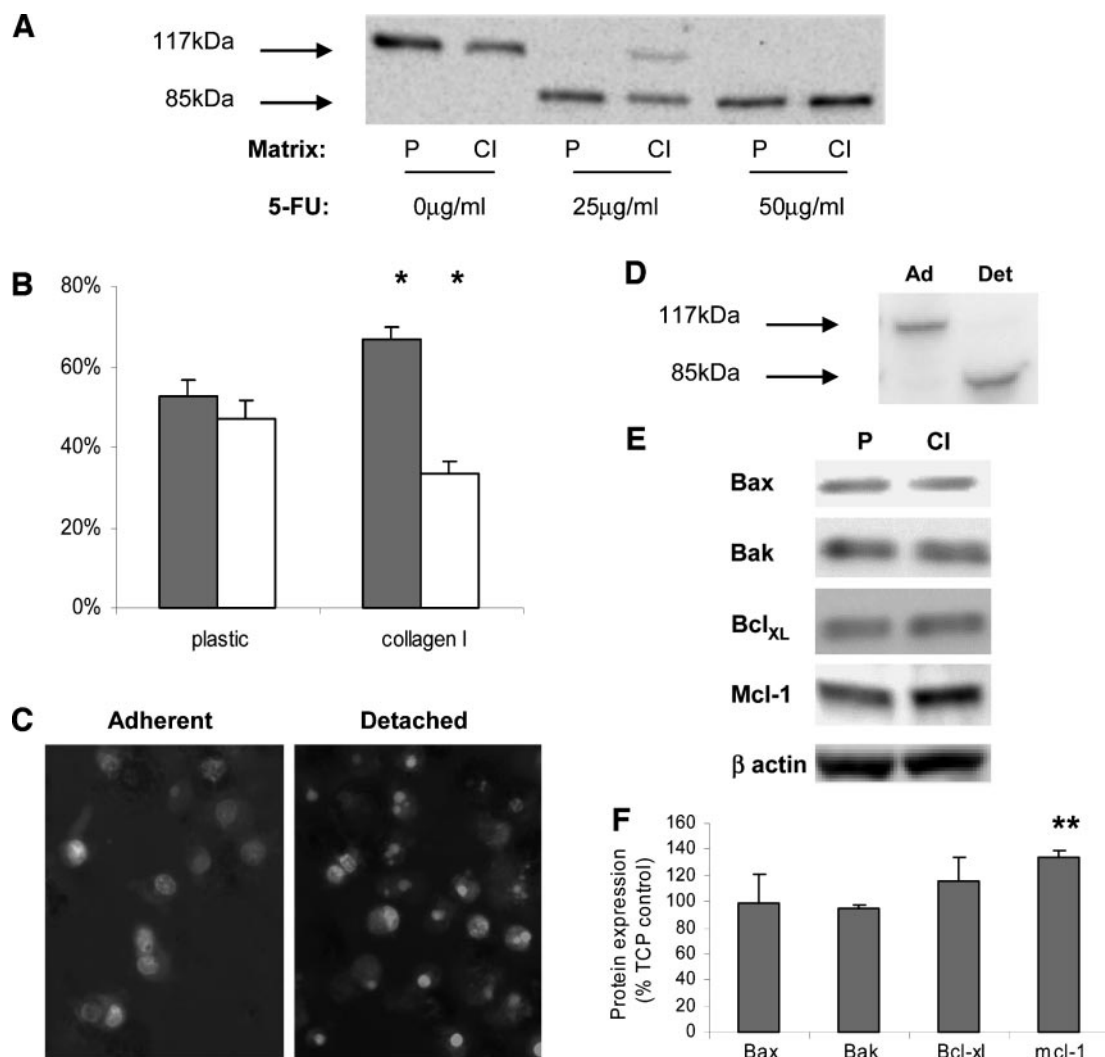
## DISCUSSION

This series of tissue culture experiments demonstrates that pancreatic cancer cells have the potential to elicit a DR rich in type I collagen and reveal underlying mechanisms that support this fibrotic response based on the ability of the cancer cells to promote PSC proliferation and collagen synthesis. Crucially, the cancer cells in turn derive a growth and survival advantage from type I collagen that predominates in the DR. The interactions among cancer cells, PSCs, and type I collagen, although not entirely uniform, demonstrated a series of common themes, suggesting that this is likely to represent the situation *in vivo*, where malignant cells comprising tumors demonstrate numerous genotypic/phenotypic variations. Specifically, both AsPC-1 cells derived from metastases and MIA PaCa-2/Panc-1 cells derived from a primary tumor consistently derived a growth and survival advantage from type I collagen. This evidence indicates that the DR in pancreatic cancer is ultimately detrimental to the host.

The close spatial arrangement of PSCs and pancreatic cancer cells indicates the potential for the paracrine interaction demonstrated *in vitro* to occur *in vivo*. The cancer cells provided a consistent stimulus to PSC proliferation *in vitro* and, as such, provide an explanation regarding the numerous PSCs in PDAC (8, 26). The stimulus to PSC proliferation would in itself increase overall synthesis of fibrillar collagen, but there was also some evidence that pancreatic cancer cells regulated PSC collagen synthesis at a transcriptional and posttranscriptional level. Although there was a suggestion that two cell lines increased procollagen I mRNA, measurement of PSC collagen synthesis with the [<sup>3</sup>H]hydroxyproline assay provided strong evidence that AsPC-1 cells increase PSC collagen synthesis. Indeed, similar evidence of the relative ability of the cell lines to elicit a DR was observed after orthotopic transplantation of pancreatic cancer cells in nude mice, in which AsPC-1 cells provoked a more pronounced DR than Panc-1 cells (27). Although the cell lines secrete TGF-β1 and appeared to stimulate expression of

**Fig. 9** Effect of type I collagen proliferation of the cancer cells. **A**, [<sup>3</sup>H]thymidine incorporated by  $1.0 \times 10^4$  MIA PaCa-2, Panc-1, or AsPC-1 cells cultured on TCP or type I collagen over 16 hours. Scintillation counts were normalized to total DNA present in wells at the end of the assay to account for potential variation in adhesion, giving results in units of counts per minute per nanogram of DNA. Data are presented as a percentage of TCP controls, which were given arbitrary values of 100%. The bars represent the mean  $\pm$  SE of six independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (with respect to the results obtained on TCP). **B**, example of acquired dot plot of cells sorted according to their stage in the cell cycle using FACS analysis (by DNA content on the X axis and FITC-labeled BrdUrd incorporated during pulse labeling on the Y axis). The gate used to identify BrdUrd-labeled cells is shown. **C**, histograms generated by DNA content of BrdUrd-labeled cells that were cultured on TCP and type I collagen. These histograms are representative of three separate experiments using Panc-1 cells (similar results were obtained with AsPC-1 cells).





**Fig. 10** Effect of type I collagen on apoptosis of AsPC-1 cells exposed to 5-FU. **A**, Western analysis of PARP (an enzyme cleaved by caspases during apoptosis yielding an 85-kDa band). AsPC-1 cells underwent dose-dependent apoptosis in response to 5-FU, but a relative reduction in PARP cleavage was observed in cells treated with 25 μg/mL 5-FU on type I collagen compared with TCP. This relative protection disappeared at higher concentrations of 5-FU (the image is representative of three separate experiments). **B**, This protection from apoptosis was observed in AsPC-1 cells treated with 5-FU while being cultured on type I collagen. The relative number of adherent cells (■) was counted and compared with those that had undergone apoptosis and become detached (□). *Bars* represent the mean of five independent experiments ± SE. \*,  $P < 0.05$  with respect to results obtained on TCP. **C**, Detached cells were apoptotic as judged by nuclear morphology after staining with acridine orange. **D**, Western analysis of PARP indicated that it was uncleaved in the adherent cells but cleaved in the detached cells, confirming the apoptotic nature of the detached cells. **E**, Western analysis of protein regulators of mitochondrial cytochrome *c* release during apoptosis revealed up-regulation of antiapoptotic mcl-1 proteins by type I collagen, consistent with changes observed in apoptosis (the image is representative of three separate experiments). β-Actin was used as a loading control. **F**, Densitometric analysis was conducted on three separate Western blots, and protein expression on type I collagen is shown as a percentage of that obtained in cells cultured on TCP (\*\*,  $P < 0.01$ ).

procollagen I mRNA, the process of matrix deposition is complex and also regulated by proteases. MMP-2 is consistently expressed in pancreatic cancer and correlates with a worse prognosis (28–30). Likewise, TIMP-1 is widely expressed in pancreatic cancer and inhibits most MMPs (31). MMP-2 and TIMP-1 were therefore studied as representatives of MMPs and TIMPs to determine their relative expression in pancreatic cancer and how this may impact on the DR. *In vitro* experiments indicate that PSCs rather than pancreatic cancer cells are quantitatively the main source of MMP-2 and TIMP-1. Furthermore,

the relative abundance of TIMP-1 over MMP-2 would also promote the accumulation of extracellular matrix in pancreatic cancer. Cancer cells and PSCs do not necessarily occur in equal numbers; neoplasia and malignant cells may therefore play a more significant role in tumor matrix turnover *in vivo* than these experiments suggest.

The effect of type I collagen on the growth and survival of pancreatic cancer cells *in vivo* remains unknown. The role of stromal reaction associated with malignancy has long been a matter of debate (32). The clonogenic assays demonstrated that

type I collagen confers a survival advantage to MIA PaCa-2, Panc-1, and AsPC-1 cells. The clonogenic assay is analogous to a cycle of chemotherapy with surviving cells repopulating the tumor, thus selecting chemoresistant clones. Additional studies indicated that type I collagen influenced clonogenicity by regulating proliferation and apoptosis of the pancreatic cancer cells. Each cell line derived a marked proliferative advantage from type I collagen, the dominant matrix component of the stromal reaction in pancreatic cancer. Type I collagen has also been shown to promote proliferation of prostate cancer cells by signaling via mitogen-activated protein kinase and phosphatidylinositol 3'-kinase, culminating in increased cyclin D1 expression and cell cycle progression (33, 34).

AsPC-1 cells demonstrated a less marked proliferative response but became resistant to apoptosis when cultured on type I collagen. Mcl-1, a bcl-related protein that inhibits the release of cytochrome *c* from mitochondria during apoptosis, was subject to regulation, consistent with the changes in apoptosis observed on type I collagen. Bcl-2-related proteins have also been shown to be regulated by mitogen-activated protein/extracellular signal-regulated kinase pathways in pancreatic cancer cells, and manipulation of this pathway has shown that it is important in regulating 5-FU-induced apoptosis (35, 36). Although the precise signaling pathways and molecular control underlying the growth-promoting effects of type I collagen in PDAC have yet to be determined, their combination gives pancreatic cancer cells a potentially real survival advantage in the clinical setting.

As well as the effects on proliferation and apoptosis, fibrillar collagen has also been implicated in migration and invasion of pancreatic cancer cells (37). Type I collagen reduces E-cadherin expression via a Src-dependent mechanism, which is consistent with the observation that pancreatic cancer cells cultured in type I collagen gels form only loose aggregates (37, 38). In contrast, type IV collagen and other basement membrane components including laminin and certain proteoglycans inhibited proliferation of pancreatic cancer cells and promoted redifferentiation to a more regulated cellular phenotype (38–41). Type IV collagen has also been shown to inhibit proliferation and migration and reduce angiogenesis and MMP expression in other tumor cells (42–45). Hence, the shift in expression of matrix in the DR, with type I collagen predominating, potentially promotes a number of phenotypic qualities that enhance tumor development, growth, and spread of PDAC.

We conclude that there is a potentially deleterious tissue response in pancreatic cancer, in which pancreatic cancer cells elicit the formation of a DR, which has the potential to promote a number of phenotypic qualities that would ultimately be detrimental to the host. These findings complement growing recognition of the numerous, important roles that tumor stroma plays in angiogenesis, local invasion, metastasis, chemosensitivity, and tumorigenesis in neoplasia (16, 46–51).

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