Insulin-like Growth Factor-I Receptor Mediates the Prosurvival Effect of Fibronectin

We recently showed that extracellular matrix (ECM) proteins, which are abundant in desmoplastic pancreatic tumor, are as potent as growth factors in inhibiting apoptosis in pancreatic cancer (PaCa) cells. Here we show that fibronectin, a major ECM component, engages insulin-like growth factor-I receptor (IGF-IR) to inhibit PaCa cell death. We found that fibronectin-induced protection from apoptosis is fully mediated by IGF-IR and is independent of IGF-I. Pharmacologic and molecular inhibitions of IGF-IR stimulated apoptosis and prevented the pro-survival effect of fibronectin in PaCa cells. Our data indicate that fibronectin protects from apoptosis through trans-activation of IGF-IR. We showed that fibronectin stimulated complex formation between its receptor β3 integrin and protein-tyrosine phosphatase SHP-2. This process of complex formation, in turn, prevents SHP-2 from dephosphorylating IGF-IR resulting in sustained phosphorylation of IGF-IR and leading to the downstream activation of Akt kinase, up-regulation of antiapoptotic Bcl-xl, and inhibition of apoptosis. Among ECM proteins tested only fibronectin and laminin but not vitronectin and collagen I stimulated trans-activation of IGF-IR. Interaction of fibronectin with β3 but not β1 integrin receptors mediates the survival pathway. In contrast, fibronectin-induced adhesion is mediated through β1 integrin receptor and is IGF-IR-independent. Thus, our results indicate that the prosurvival effect of fibronectin in PaCa cells is mediated by trans-activation of IGF-IR induced by the β3 integrin receptor. The data suggest IGF-IR as a key target for prevention of the prosurvival effects of ECM proteins and growth factors in pancreatic cancer.

Pancreatic adenocarcinoma is a fibrotic tumor, which is characterized by a high level of extracellular matrix (ECM) proteins. ECM mediates adhesion and invasiveness of cancer cells. Cell survival also depends on adequate connections with ECM proteins through specific integrin receptors. We recently showed that ECM proteins are key mediators of PaCa cell survival (4, 5) and that detachment from ECM stimulates PaCa cell death. The mechanisms through which ECM proteins protect PaCa cells from death are not fully understood.

Fibronectin is a major ECM protein of pancreatic adenocarcinoma; its receptors, β3 and β1 integrins, are up-regulated in pancreatic cancer cells (6). Fibronectin stimulates invasion and adhesion and markedly increases survival of pancreatic cancer cells (7, 8).

The importance of fibronectin and other extracellular matrix proteins in pancreatic cancer is underscored by several findings. Pancreatic adenocarcinoma is one of the most lethal of human cancers with a 5-year survival rate of less than 5% (9). This cancer has a characteristic of marked desmoplasia, which is a remarkable increase in extracellular matrix that infiltrates and envelopes the cancer (10). Importantly there is at least one report that demonstrates that greater amounts of intratumor extracellular matrix in pancreatic cancer patients are correlated with more aggressive disease and more rapid death (11). Finally recent studies in animal models of pancreatic cancer show that tumors grow more rapidly when the fibroblastic cells of the cancer called stellate cells are included in inocula with cancer cells (12). The addition of the stellate cells results in the experimental tumors taking on the characteristics of the human cancer with the extracellular matrix infiltrating and enveloping the cancer cells.

In addition, another powerful mediator of PaCa cell survival is insulin-like growth factor-I (IGF-I). Both IGF-I and IGF-I receptor (IGF-IR) are overexpressed in human pancreatic tumors as well as in pancreatic cancer cell lines (13, 14). Blockade of the IGF-IR by a dominant negative inhibitor suppresses tumorigenicity both in vitro and in vivo and increases sensitivity of pancreatic tumors to radiation and chemotheraphy-induced apoptosis.

The key event in IGF-I-induced signaling is phosphorylation of IGF-I receptor kinase followed by the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which mediates the antiapoptotic effect of IGF-I (4). Another important

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Pancreatic adenocarcinoma is the fourth most common cause of death in western countries with almost the same rate of incidence and mortality per year (1, 2). The disease is very resistant to radio- and/or chemotherapy. One reason for that is the resistance of pancreatic cancer (PaCa) cells to apoptosis (3).

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2 The abbreviations used are: PaCa, pancreatic cancer; ECM, extracellular matrix; IGF, insulin-like growth factor; IGF-IR, insulin-like growth factor-I receptor; AMC, aminomethylcoumarin; p-, phospho-; polyHEMA, poly(2-hydroxyethyl methacrylate); siRNA, small interfering RNA; AnV, Annexin V; PI, propidium iodide; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; PI3K, phosphatidylinositol 3-kinase.
Fibronectin and IGF-I Receptor in Pancreatic Cancer

FIGURE 1. Inhibition of IGF-IR prevents the antiapoptotic effect of fibronectin in pancreatic cancer cells. MIA PaCa-2 (A–D) and PANC-1 (B) cells were cultured on polyHEMA or fibronectin for 48 h in the presence or absence of IGF-I (100 ng/ml) (A) or IGF-I inhibitor AG1024 (10 μM) (B–D). A and B, intranuclear DNA fragmentation was measured using the Cell Death Detection ELISA kit. C, caspase-3 activity was measured with fluorogenic assay using the DEVD-AMC as a substrate. D, cells were stained with AnV and PI and analyzed by flow cytometry; AnV⁻/PI⁻ and AnV⁻/PI⁺ were considered dead cells, and AnV⁺/PI⁻ were considered living cells. A, B, and C, values are normalized to those for polyHEMA. A–D, values are means ± S.E. (n = 3), *, p < 0.05 versus polyHEMA; #, p < 0.05 versus fibronectin without inhibitor (ANOVA).

step in IGF-I-induced signaling is phosphorylation of adapter protein SHPS-1 leading to the recruitment of Src homology 2-containing phosphotyrosine phosphatase SHP-2. SHP-2 is a negative regulator of IGF-IR signaling. It dephosphorylates IGF-IR resulting in the inhibition of its signaling (15).

Very little is known on the role of IGF-IR in the effects of ECM proteins. In contrast the contribution of integrins in the signaling induced by exogenous IGF-I cells was established in several publications mostly in smooth muscle cells (16, 17). In these cells IGF-I-induced phosphorylation of β3 integrins results in recruitment of phosphatase SHP-2, which is required for proper IGF-IR phosphorylation. Both β3 and SHP-2 knockdowns result in the inhibition of functional IGF-1 responses, proliferation, and migration (16).

Here we show that prosurvival effects of fibronectin are mediated by IGF-IR trans-activation. Vitronectin and collagen, which had no effect on cell survival, do not trans-activate IGF-IR. Adhesion is not required for fibronectin-induced IGF-IR trans-activation. Furthermore IGF-IR is not involved in fibronectin-induced adhesion of PaCa cells. Fibronectin trans-activates IGF-IR by preventing receptor dephosphorylation. Fibronectin stimulates complex formation between its receptor β3 integrin and protein-tyrosine phosphatase SHP-2. This in turn prevents SHP-2 from dephosphorylating IGF-IR resulting in sustained phosphorylation of IGF-IR and leading to the downstream activation of Akt kinase, up-regulation of anti-apoptotic Bcl-xL, and inhibition of apoptosis.

Our results indicate that interaction between IGF-IR and β3 integrin plays a different role in the effects of fibronectin and IGF-1. Inhibition of apoptosis by fibronectin is mediated by SHP-2 immobilization away from IGF-IR and is mimicked by SHP-2 knockdown. In contrast IGF-I signaling as well as downstream proliferation and migration are inhibited by SHP-2 knockdown (16).

EXPERIMENTAL PROCEDURES

Reagents—Ac-DEVD-AMC (Ac-Asp-Glu-Val-Asp-aminoethylcoumarin) was from Peptide Institute, Inc. (Osaka, Japan). Antibodies against IGF-IR, SHP-2, Bcl-xL, and insulin blocking peptide were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against α2 and β3 integrins and the blocking antibodies against β1 and β3 integrins were from Chemicon International (Temecula, CA). αV antibody and IGF-I blocking antibody were from Calbiochem, phospho-Akt (p-Akt) antibody was from Cell Signaling Technology (Danvers, MA), p-IGF-IR antibody was from Upstate, Inc. (Lake Placid, NY), and platelet-derived growth factor blocking antibody was from R&D Systems (Minneapolis, MN). All other reagents were from Sigma.

Cell Culture—Human pancreatic adenocarcinoma cell lines, the poorly differentiated MIA PaCa-2 and the slightly differentiated PANC-1, were obtained from the American Type Culture Collection (Manassas, VA). MIA PaCa-2 and PANC-1 cells were grown in 1:1 Dulbecco’s modified Eagle’s medium/F-12 medium (Invitrogen) supplemented with 15% fetal bovine serum, 4 ml/glucose, and antibiotic/antimycotic solution (Omega Scientific, Tarzana, CA). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO2 and were used between passages 4 and 12.

MIA PaCa-2 and PANC-1 cells were plated at a density of 2 × 10⁶/ml on 100-mm culture dishes coated with either poly(L-hydroxyethyl methacrylate) (polyHEMA), fibronectin, collagen I, vitronectin, or soluble fibronectin (Arg-Gly-Asp); cultured for up to 48 h in serum-free Dulbecco’s modified Eagle’s medium/F-12 medium; collected; and processed for the specified analyses. PolyHEMA prevents both cell attachment on plastic and endogenous cell matrix deposition (18). Plates were coated twice with 3 ml of polyHEMA dissolved in 95% ethanol (10 mg/ml), allowed to dry, and washed twice with phosphate-buff-
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FIGURE 2. Molecular knockdown of IGF-IR inhibits the prosurvival effect of fibronectin. MIA PaCa-2 and PANC-1 cells were cultured on polyHEMA or fibronectin for 48 h. A and B, cells were transfected with 200 nM IGF-IR antisense (AS) or scrambled (Sc) oligonucleotides and then cultured for 48 h on polyHEMA or fibronectin. C, cells were transfected with 200 nM IGF-IR or control siRNA and then cultured for 48 h on polyHEMA or fibronectin. Internucleosomal DNA fragmentation was measured using the Cell Death Detection ELISA kit. Values are normalized to those for cells transfected with scrambled oligonucleotides (A and B) or control siRNA (C) and cultured on polyHEMA (A and C) or on fibronectin (B). Values are means ± S.E. (n = 3), *, p < 0.05 versus control transfection on polyHEMA; #, p < 0.05 versus control transfection on fibronectin (ANOVA). Transfection efficiency was assessed by Western blot. Blots were stripped and reprobed for actin to confirm equal loading.

Western Blot Analysis—Cells were incubated in a lysis buffer (0.5 mM EDTA, 150 mM NaCl, 50 mM Tris, 0.5% Nonidet P-40, pH 7.5) for 30 min at 4 °C. The lysis buffer was supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitors pepstatin, leupeptin, chymostatin, antipain, and aprotinin, each at 5 μg/ml. Cell lysates were centrifuged for 10 min at 13,000 × g. Supernatants were collected, and proteins were separated by SDS-PAGE (Invitrogen) and electrophoretically transferred to nitrocellulose membranes. Nonspecific binding was blocked with 5% milk in Tris-buffered saline (4 mM Tris base, 100 mM NaCl, pH 7.5). Membranes were washed in Tris-buffered saline containing 0.05% Tween 20 and incubated for 2 h with the indicated primary antibodies and then for 1 h with horseradish peroxidase-conjugated secondary antibody. Blots were developed with the SuperSignal chemiluminescent substrate (enhanced chemiluminescence) (Pierce).

Immunoprecipitation—Immunoprecipitation was done according to the Catch and Release Reversible Immunoprecipitation System protocol (Upstate, Inc.). Cell lysates were prepared as mentioned in the Western blot protocol. They were then centrifuged for 10 min at 13,000 × g. Supernatants were collected, and protein concentration was measured. The supernatant was added to the antibody capture affinity ligand and the specific antibody and incubated in a Catch and Release spin column at room temperature for 1 h under continuous shaking. The column was then washed three times with the wash buffer (10% Nonidet P-40, 2.5% deoxycholic acid, 150 mM imidazole, pH 7.4). The immunoprecipitate was then eluted with Tris-buffered saline containing 0.05% Tween 20 and incubated for 2 h with the indicated primary antibodies and then for 1 h with horseradish peroxidase-conjugated secondary antibody. Blots were developed with the SuperSignal chemiluminescent substrate (enhanced chemiluminescence) (Pierce).

Measurements of Apoptosis—Internucleosomal DNA fragmentation was measured by using the Cell Death Detection...
Figure 3. Fibronectin stimulates IGF-IR phosphorylation in pancreatic cancer cells. MIA PaCa-2 (A and B) and PANC-1 (C and D) cells were cultured on polyHEMA or fibronectin for the indicated times. The p-IGF-IR level was measured by immunoblotting. Blots were stripped and reprobed for total IGF-IR level (t-IGF-IR) and for actin to confirm equal loading. Blots were quantified by densitometry, and values in B and D are means of the ratio of the intensities of p-IGF-IR/total IGF-IR ± S.E. (n = 3). Values are normalized to those for polyHEMA at 2 min. *p < 0.05 versus polyHEMA at each time (B) or at 2 min (D) (ANOVA). ′ minutes.

ELISAPlus kit (Roche Applied Science) according to the manufac- turer’s instructions.

Phosphatidylserine externalization was analyzed with the Annexin-V-FLUOS Staining kit from Roche Applied Science as we described before (8). Cells were collected and resuspended at a density of 1 × 10⁶ cells in 500 μl of binding buffer containing 2 μl of Annexin V (AnV) and 1 μl propidium iodide (PI), incubated in the dark for 30 min at room temperature, and analyzed by flow cytometry.

Effector caspase (DEVDase) activity was measured by a fluo- rogenic assay in whole cell lysates using DEVD-AMC as a sub- strate as we described before (8). The lysate (50–100 μg of protein) was incubated with 10 μM substrate in a reaction buffer (25 mM HEPES (pH 7.5), 10% sucrose, 0.1% CHAPS, 10 mM dithiothreitol) at 37°C. Caspase substrate cleavage releases AMC, which emits fluorescent signal with 380 nm excitation and 440 nm emission. Fluorescence was calibrated using a standard curve for AMC.

Adhesion Assay—96-well plates were coated with fibronectin. The adhesion assay was then performed as described previously (20) with minor changes. Briefly the adhesive cells were stained with crystal violet, and the optical density was measured at 550 nm.

IGF-I Measurement—IGF-I level was measured using the ELISA kit according to the manufacturer’s instructions (Antigenix America Inc.).

Statistical Analysis—Results are expressed as means ± S.E. from at least three independent experiments. Statistical analysis using unpaired Student’s t test was done to analyze differences between two conditions. One-way analysis of variance (ANOVA) and multiple comparisons using the Student-Newman-Keuls test were used to analyze differences between several conditions.

RESULTS

Pharmacological and Molecular Inhibitions of IGF-IR Prevent the Antiapoptotic Effect of Fibronectin in Pancreatic Cancer Cells—In accord with our previous publications (7, 8), fibronectin and IGF-I decreased apoptosis in pancreatic cancer cells (Fig. 1A). Importantly, combination of fibronectin and IGF-I did not further decrease apoptosis in comparison with fibronectin or IGF-I alone (Fig. 1A) suggesting that the prosurvival pathways of IGF-I and fibronectin overlap possibly through activating the same prosurvival target such as IGF-IR. To explore the role of IGF-IR activation in the prosurvival effect of fibronectin, we showed that the inhibitory effects of fibronectin on different parameters of apoptosis were completely prevented by the IGF-IR-specific inhibitor AG1024 (Fig. 1, B–D). Of note, AG1024 belongs to the tyrphostin family; it selectively inhibits the IGF-IR autophosphorylation (21). In particular, in the presence of AG1024, fibronectin did not decrease DNA fragmentation and caspase-3 activity and did not stimulate the survival rate in PaCa cells (Fig. 1, B–D). Similarly, transfection of MIA PaCa-2 and PANC-1 cells with IGF-IR antisense oligonucleotides significantly increased DNA fragmentation in cells cultured on fibronectin (Fig. 2A). Furthermore, the prosurvival effect of fibronectin was abolished in cells transfected with siRNA against IGF-IR (Fig. 2B).

Fibronectin Stimulates IGF-IR Phosphorylation: the Effect of Fibronectin on IGF-IR Phosphorylation Correlates with Its Effect on Apoptosis—To determine the effect of fibronectin on the phosphorylation level of IGF-IR we cultured MIA PaCa-2 and PANC-1 cells for the indicated times on polyHEMA or fibronectin (Fig. 3). PolyHEMA prevents cells from attaching and also prevents extracellular matrix deposition (18). Fig. 3 shows that fibronectin time-dependently increased the level of IGF-IR phosphorylation in MIA PaCa-2 (Fig. 3, A and B) and PANC-1 (Fig. 3, C and D) cells suggesting that IGF-IR is trans-activated by fibronectin. The increase in IGF-IR phosphorylation was already evident at 2 min and was maximal at 5 min. It reached 6-fold in MIA PaCa-2 and 3-fold in PANC-1 cells and was sustained for up to 24 h (Fig. 3, B and D). There were no changes in IGF-IR phosphorylation in cells cultured on polyHEMA or polylsine (not shown) in the same conditions.
To test the effect of other matrices on IGF-IR phosphorylation, we cultured MIA PaCa-2 cells on polyHEMA, plastic, collagen I, vitronectin, laminin, and fibronectin and on plastic in the presence of soluble fibronectin fragments (Arg-Gly-Asp) for 5 min and measured the phosphorylation level of IGF-IR. Fig. 4A shows that culturing on plastic, collagen I, and vitronectin did not increase phosphorylation of IGF-IR as compared with that on polyHEMA. Similarly neither vitronectin nor collagen I affected PaCa cell survival (Fig. 4C). Up-regulation of phosphorylated IGF-IR was only observed in cells cultured on fibronectin and to a lesser extent on laminin and soluble fibronectin (Fig. 4A). These three matrices were able to support PaCa cell survival (Fig. 4C). Thus, the ability of ECM proteins to inhibit apoptosis correlates with their ability to increase the level of IGF-IR phosphorylation (Fig. 4, A and C). Of note, integrin receptors for vitronectin and collagen I are present in MIA PaCa-2 cells.
Results with soluble fibronectin indicate that fibronectin stimulates IGF-IR phosphorylation even in non-adhered cells.

**The Fibronectin-induced Phosphorylation of IGF-IR and Protection from Apoptosis Are Independent of IGF-I**—The effects of fibronectin on IGF-IR phosphorylation could be due to the increased level of IGF-I in extracellular medium and its interaction with IGF-IR. To determine the role of IGF-I in the effects of fibronectin we used several approaches. First we measured the effect of fibronectin on the extracellular level of IGF-I in MIA PaCa-2 and PANC-1 cells. Fig. 5A shows that in cells cultured on polyHEMA or on 2 μg/cm² or indicated doses of fibronectin for 5 min or for the indicated time in the presence or absence of the β1 and β3 blocking antibodies (10 μg/ml), the p-IGF-IR level was analyzed by immunoblotting. Blots were stripped and reprobed for total-IGF-IR (t-IGF-IR) to confirm equal loading. Blots were quantified by densitometry, and values are means of the ratio p-IGF-IR/total IGF-IR ± S.E. (n = 3). Values were normalized to those of polyHEMA. A and B, adhesive cells were stained with crystal violet, and the optical density was read at 550 nm. Values are means ± S.E. (n = 31); *, values were statistically significantly different from those for polyHEMA (A and D); #, values were statistically significantly different from those for fibronectin without antibody (D) (ANOVA; p < 0.05).

Other ligands, which could directly or indirectly activate IGF-IR, include insulin, IGF-II, and platelet-derived growth factor (22, 23). Neutralization of insulin, IGF-II, and platelet-derived growth factor did not block the fibronectin-induced increase in IGF-IR phosphorylation, suggesting against their involvement (Fig. 5E). Furthermore neither Src kinase inhibitor PP2 nor Src kinase siRNA affected the fibronectin-induced IGF-IR phosphorylation, suggesting against involvement of Src kinase (not illustrated).

Thus, our data suggest that IGF-I is not a critical mediator of the effects of fibronectin on IGF-IR phosphorylation. We cannot exclude, however, that IGF-I at very low concentrations
might be present in extracellular medium and may contribute to the basal IGF-IR phosphorylation. Our data also do not exclude involvement of IGF-I-binding proteins in the effects of fibronectin on IGF-I.

**β3 Integrin Mediates the Fibronectin-induced Phosphorylation of IGF-IR, whereas Only β1 Integrin Is Involved in Cell Adhesion**—To assess the involvement of β3 and β1 integrins in the observed IGF-IR phosphorylation, we applied neutralizing anti-β3 and anti-β1 antibodies that were shown before to prevent interaction of fibronectin with the corresponding integrins (24, 25). The fibronectin-induced increase in phosphorylation of IGF-IR was abolished in cells cultured on fibronectin in the presence of the β3 blocking antibody (Fig. 6C). In contrast, blockade of β1 integrin increased IGF-IR phosphorylation (Fig. 6C). These data indicate that IGF-IR trans-activation is mediated by β3 but not β1 integrins. They further suggest that β1 inhibition potentiates interaction of fibronectin with β3 integrin. The increase in IGF-IR phosphorylation induced by β1 blocking antibody (Fig. 6C) could be due to less competition between β1 and β3 integrins to interact with fibronectin.

Next we analyzed the role of adhesion in the effects of fibronectin on IGF-IR phosphorylation. Fig. 6A shows that fibronectin dose-dependently increases adhesion of MIA PaCa-2 and PANC-1 cells. The effect of fibronectin on adhesion was also time-dependent (Fig. 6B). Neutralizing antibody against β3 integrin produced no effect on adhesion of PANC-1 cells and only delayed adhesion of MIA PaCa-2 cells at early times. By contrast, antibody against β1 integrin inhibited adhesion in both MIA PaCa-2 and PANC-1 cells at all times tested (Fig. 6B). These data indicate that β1 rather than β3 integrin mediates PaCa cell adhesion.

Fig. 6D compares the effects of neutralizing anti-β3 and anti-β1 antibodies on the adhesion and on IGF-IR phosphorylation in MIA PaCa-2 cells cultured for 5 min on fibronectin. Inhibition of β3 integrin slightly decreased adhesion but markedly prevented IGF-IR phosphorylation, whereas inhibition of β1 integrin greatly inhibited adhesion but produced no decrease in IGF-IR phosphorylation (Fig. 6D).

These data suggest that the fibronectin-induced activation of IGF-IR is not associated with cell adhesion.

**SHP-2 Mediates the Regulation of IGF-IR Phosphorylation by Fibronectin**—The fibronectin-induced increase in phosphorylation level of IGF-IR could be mediated by several mechanisms. They include up-regulation of the IGF-IR protein level, stimulation of IGF-IR kinase activity, or inhibition of the IGF-IR dephosphorylation. Neither transcriptional blocker actinomycin D (2.5 μg/ml) nor the inhibitor of protein synthesis cycloheximide (10 μg/ml) prevented the phosphorylation of IGF-IR by fibronectin (not shown) suggesting that the increase in phosphorylation was not due to the increased protein level of IGF-IR. The protein phosphatase inhibitor bisperoxyvanadate stimulated IGF-IR phosphorylation in cells cultured on polyHEMA indicating involvement of phosphatases (Fig. 7A). Importantly in the presence of bisperoxyvanadate, fibronectin did not further stimulate phosphorylation suggesting that the fibronectin effect is mediated by phosphatase inhibition (Fig. 7A).

SHP-2 regulates IGF-IR signaling in other cells (17). Therefore, we tested whether phosphatase SHP-2 regulates IGF-IR phosphorylation in PaCa cells. Transfection with SHP-2 antisense oligonucleotides increased the phosphorylation level of IGF-IR in MIA PaCa-2 cells cultured on polyHEMA suggesting that SHP-2 regulates phosphorylation of IGF-IR. Importantly in cells transfected with SHP-2 antisense oligonucleotides, fibronectin did not further stimulate IGF-IR phosphorylation.
suggesting that the fibronectin-induced phosphorylation of IGF-IR is mediated by SHP-2 (Fig. 7B).

We further showed that β3 integrin and SHP-2 are physically associated in pancreatic cancer cells. That is, Western blot analysis detected SHP-2 in β3 integrin immunoprecipitates, and vice versa, antibody recognized β3 integrin in SHP-2 immunoprecipitates. Co-immunoprecipitation of β3 integrin with SHP-2 was significantly greater in pancreatic cancer cells cultured on fibronectin than in those cultured on polyHEMA (Fig. 7, C and D). Of note, unrelated immunoglobulin of the same species did not immunoprecipitate β3 integrin or SHP-2, confirming specificity of the assay.

The results of Fig. 7 suggest SHP-2 as a mediator of the fibronectin-induced up-regulation of IGF-IR phosphorylation. Upon binding to fibronectin, β3 integrins recruit and immobilize SHP-2 preventing it from dephosphorylating IGF-IR.

β3 Integrin and SHP-2 Mediate the Antiapoptotic Effect of Fibronectin—To confirm that the antiapoptotic effect of fibronectin is mediated through β3 integrin we cultured MIA PaCa-2 cells on fibronectin in the presence or absence of β3 integrin blocking antibody. Fig. 8A demonstrates that β3 integrin blocking antibody prevents the fibronectin-induced inhibition of DNA fragmentation. These data are in agreement with the results in Fig. 6C demonstrating that blockade of β3 integrin prevented the fibronectin-induced IGF-IR phosphorylation. In Fig. 7, we showed that fibronectin stimulates physical association of protein-tyrosine phosphatase SHP-2 with β3 and that knocking down SHP-2 with antisense oligonucleotides in cells cultured on polyHEMA results in increased phosphorylation of IGF-IR similar to that induced by fibronectin. Fig. 8B shows that SHP-2 antisense transfection decreased DNA fragmentation in cells cultured on polyHEMA to the same extent as fibronectin. In cells transfected with SHP-2 antisense oligonucleotides and cultured on fibronectin there was no further decrease in apoptosis (not shown). The combined results of Figs. 6C, 7, and 8, A and B, provide evidence that the antiapoptotic effect of fibronectin is mediated through β3 integrin ligation leading to complex formation between β3 and SHP-2 that results in increased IGF-IR phosphorylation.

Exogenous IGF-I was shown to stimulate β3 integrin recruitment in smooth muscle cells through β3 phosphorylation by Src kinase. In contrast, in the pancreatic cancer cells fibronectin stimulates β3 integrin-SHP-2 complex formation in the absence of Src-induced β3 integrin phosphorylation because Src kinase inhibitor PP2 did not affect the fibronectin-induced IGF-IR phosphorylation (not illustrated).

IGF-IR Mediates the Downstream Pathway of the Fibronectin-induced Antiapoptotic Effect—The fibronectin-induced protection from apoptosis in pancreatic cancer cells is mediated by the PI3K/Akt pathway (19). Indeed inhibition of PI3K by its inhibitor LY294002 increased DNA fragmentation in MIA PaCa-2 cells cultured on fibronectin (Fig. 8C). The fibronectin-induced Akt phosphorylation was abolished in the presence of the inhibitor of IGF-IR, AG1024, suggesting involvement of IGF-IR in Akt phosphorylation (Fig. 8D). LY294002 produced similar inhibition of Akt phosphorylation (Fig. 8D).

FIGURE 8. Signaling mediating the prosurvival effect of fibronectin via IGF-IR trans-activation. MIA PaCa-2 cells were cultured on polyHEMA and fibronectin for 48 h. Cells were cultured in the presence or absence of the β3 integrin blocking antibody (10 μg/ml) (A), the IGF-IR inhibitor AG1024 (10 μM) (D and E), or the PI3K inhibitor LY294002 (50 μM) (C and D). In B and F, cells were transfected with 200 nM SHP-2 antisense (As) or scrambled (Sc) oligonucleotides and cultured on polyHEMA or fibronectin for 48 h. Levels of p-Akt and BclxL (D–F) and transfection efficiency (B and F) were assessed by Western blot. Blots were stripped and reprobed for actin to confirm equal loading. In A–C, internucleosomal DNA fragmentation was measured using the Cell Death Detection ELISA kit. Values are normalized to those for polyHEMA (A), SHP-2 scrambled oligonucleotides on polyHEMA (B), or fibronectin alone (C). Values in A–C are means ± S.E. (n = 3). *, p < 0.05 versus polyHEMA (A); SHP-2 scrambled oligonucleotides on polyHEMA (B), and fibronectin alone (C); #, p < 0.05 versus fibronectin alone (A).

BclxL is another downstream signal in the prosurvival pathway of fibronectin. Our data indicate that inhibition of IGF-IR by AG1024 decreased the protein level of BclxL in MIA PaCa-2 cells cultured on fibronectin (Fig. 8E).

Importantly SHP-2 knockdown using antisense oligonucleotides increased Akt phosphorylation in cells cultured on polyHEMA similarly to fibronectin (Fig. 8F). Taken together, these results indicate that SHP-2 recruitment by fibronectin results in sustained IGF-IR phosphorylation followed by activation of antiapoptotic signaling in particular activation of Akt and up-regulation of BclxL.

DISCUSSION

ECM protein fibronectin and growth factor IGF-I are two major environmental factors protecting PaCa cells from death (5, 7). Our study was designed to investigate the role of IGF-IR in the antiapoptotic effect of fibronectin. We used poorly differentiated MIA PaCa-2 and moderately differentiated
PANC-1 human pancreatic ductal carcinoma cell lines, which both display K-ras and p53 mutations characteristic for pancreatic cancer (26). The present study is based on our previous findings on the effects of extracellular matrix proteins on cancer cell survival (8). Our key finding is that the prosurvival effect of fibronectin is mediated through activation of IGF-IR (Fig. 9). We used several lines of evidence to prove the involvement of IGF-IR and its phosphorylation in the antiapoptotic effect of fibronectin. We showed that fibronectin-induced recruitment and inhibition of the phosphatase SHP-2 mediates the regulation of IGF-IR phosphorylation and the downstream antiapoptotic pathway. In particular, we showed that pharmacologic inhibition as well as molecular knockdown of IGF-IR increased apoptosis in PaCa cells cultured on fibronectin. Conversely increasing IGF-IR phosphorylation in cells cultured on polyHEMA by knocking down SHP-2 mimicked the prosurvival effect of fibronectin.

Previous results show that fibronectin inhibits apoptosis through activating the PI3K/Akt pathway and up-regulating antiapoptotic Bclxl protein (27). We found that blockade of IGF-IR in cells cultured on fibronectin prevented both Akt phosphorylation and Bclxl up-regulation. These data indicate that trans-activation of IGF-IR is a key mechanism mediating the prosurvival effect of fibronectin in PaCa cells.

In contrast to fibronectin, culturing cancer cells on vitronectin or collagen I was without effect on either apoptosis rate or IGF-IR phosphorylation. On the other hand, ECM proteins that have a prosurvival effect, fibronectin, laminin, and soluble fibronectin, all stimulate IGF-IR phosphorylation suggesting that the prosurvival effect of ECM proteins in PaCa cells involves IGF-IR.

We found that the β3 integrin receptor but not major fibronectin receptor β1 mediates trans-activation of IGF-IR by fibronectin. Interestingly vitronectin, which acts via β3 integrin, does not trans-activate IGF-IR, suggesting that β3 engagement is necessary but not sufficient for IGF-IR phosphorylation.

Cell adhesion and cell survival are two major functions of fibronectin. Our data show that fibronectin-induced survival and adhesion are mediated through different mechanisms. Survival is mediated through β3-dependent IGF-IR trans-activation, whereas adhesion involves β1 and is independent of IGF-IR. Soluble fibronectin also trans-activates IGF-IR and inhibits apoptosis, indicating that adhesion is not required for the prosurvival effect of fibronectin. The inhibition of apoptosis by fibronectin fragments may contribute in supporting survival of cancer cells that are detached from the organ but still retain fibronectin fragments on their surface.

Normal epithelial and endothelial cells undergo apoptosis when deprived from ECM. This process, termed anoikis, deletes cells detached from the organ (28). In contrast, tumor cells upon detachment from the ECM are capable of evading anoikis and metastasizing to different distant organs. The mechanisms mediating anoikis are not fully understood; however, adhesion is considered a prerequisite to activating survival pathways by integrins. pp125FAK kinase, which is activated in response to adhesion (29), and integrin-linked kinase, which is recruited to adhesion sites, both have been implicated in survival signaling induced by α5β1 integrins.

We showed previously that classical anoikis does not operate in PaCa cells (8). The results of this study show that fibronectin inhibits apoptosis in PaCa cells through activation of IGF-IR and that this pathway is adhesion-independent. Of note, growth factors and IGF-I in particular are major prosurvival factors in PaCa cells (5). Thus, to inhibit apoptosis fibronectin recruits a major antiapoptotic signaling pathway in PaCa cells.
Our results indicate that fibronectin stimulated complex formation between β3 integrin and protein-tyrosine phosphatase SHP-2. This complex prevents SHP-2 from dephosphorylating IGF-IR resulting in sustained phosphorylation and activation of IGF-IR.

Whereas little is known on the role of growth factor receptors in the signaling pathways elicited by ECM proteins, the role of integrins in the effects of growth factors has been extensively studied in particular in smooth muscle cells (16, 17). The data from our study are different from those in the publications on the smooth muscle cells (16, 17, 30). Our study shows that fibronectin-induced activation of IGF-IR is independent of IGF-I in PaCa cells. In contrast, in the smooth muscle cells IGF-I is required and acts in concert with αVβ3 to stimulate IGF-IR phosphorylation. Furthermore our data indicate that fibronectin induces recruitment and immobilization of SHP-2 preventing it from dephosphorylating IGF-IR, and knocking down SHP-2 mimics the effect of fibronectin on apoptosis. In contrast, in the smooth muscle cells SHP-2 is recruited by integrin receptor and is independent of IGF-IR. We showed that fibronectin stimulated complex formation between its receptor integrin and protein-tyrosine phosphatase SHP-2. This complex prevents SHP-2 from dephosphorylating IGF-IR resulting in sustained phosphorylation and activation of IGF-IR.

In summary, we show that the prosurvival effect of fibronectin is mediated through trans-activation (phosphorylation) of IGF-IR leading to the downstream activation of Akt kinase, up-regulation of anti-apoptotic BclXL, and inhibition of apoptosis (Fig. 9). Fibronectin-induced survival and adhesion are mediated through different mechanisms. Survival is mediated through the β3 integrin receptor and IGF-IR activation and does not require adhesion, whereas adhesion involves the β1 integrin receptor and is independent of IGF-IR. We showed that fibronectin stimulated complex formation between its receptor β3 integrin and protein-tyrosine phosphatase SHP-2. This complex prevents SHP-2 from dephosphorylating IGF-IR resulting in sustained phosphorylation and activation of IGF-IR.

Our data suggest the inhibition of the IGF-IR pathway as a novel therapeutic strategy to inhibit prosurvival mechanisms induced in PaCa cells by both growth factors and ECM proteins and thus to overcome pancreatic cancer resistance to chemo- and radiation therapies. In conclusion, considering the importance of the extracellular matrix in the pathogenesis of pancreatic cancer as described in the Introduction, our results provide key information that can be used to develop treatment strategies directed at mechanisms underlying the pathobiology of the disease.

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