Extracellular Matrix Protects Pancreatic β-Cells Against Apoptosis

Role of Short- and Long-Term Signaling Pathways

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We have shown previously that culture of β-cells on matrix derived from 804G cells and rich in laminin-5 improves their function. The purpose of this study was to investigate whether this matrix protects β-cells against apoptosis and to elucidate signaling pathways involved. Matrix protected sorted rat β-cells against apoptosis under standard conditions (11.2 mmol/l glucose, 10% serum), after serum deprivation (1% serum), and in response to interleukin-1β (IL-1β; 2 ng/ml), compared with control (poly-L-lysine [pLL]). Caspase-8 activity was reduced in cells cultured on matrix, whereas focal adhesion kinase (FAK), protein kinase B (PKB, or Akt), and extracellular signal–regulated kinase (ERK) phosphorylation was augmented. Treatment (4 h) with an anti-β1 integrin antibody, with the ERK pathway inhibitor PD98059, and/or with the phosphatidylinositol 3-kinase inhibitor LY294002 augmented cell death on 804G matrix but not on pLL. In long-term assays (48 h), PD98059 but not LY294002 drastically augmented cell death on 804G matrix but did so to a lesser extent on pLL. The protein inhibitor of nuclear factor-κB (IκBα) was overexpressed in cells cultured 18 h on matrix with partial blockade by PD98059. In summary, this study provides evidence for activation of signaling pathways and gene expression by extracellular matrix leading to improved β-cell survival. *Diabetes* 53:2034–2041, 2004

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ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal–regulated kinase; FAK, focal adhesion kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; IκBα, inhibitor of nuclear factor κB; IL-1β, interleukin-1β; MAP, mitogen-activated protein; MEK1, MAP kinase/ERK kinase; NF-κB, nuclear factor-κB; PI, phosphatidylinositol; PKB, protein kinase B; pLL, poly-L-lysine; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling.

Epithelial cells deprived of matrix attachment undergo programmed cell death, a form of apoptosis termed anoikis (1). The role of cell anchorage in cell survival has been demonstrated in several cell types (reviewed in 2), including pancreatic islet cells (3). Disengagement from extracellular matrix (ECM) is known to prejudice islet cell survival, notably by inducing apoptosis and necrosis (4). It has been reported that reestablishment of appropriate cell-matrix contacts reduces cell death (3,5); however, the molecular basis for such pro-survival signaling remains largely unknown. Recent evidence implicates integrins in mediating pro-survival signals emanating from the ECM (reviewed in 6). Several signal transduction components activated by integrins, including focal adhesion kinase (FAK) (7,8), phosphatidylinositol (PI) 3-kinase (9,10), and the mitogen-activated protein (MAP) kinase/extracellular signal–regulated kinase (ERK) (reviewed in 11) have been implicated in mechanisms underlying anoikis (reviewed in 12). Recently, it has emerged that PI 3-kinase and its downstream effector protein kinase B (PKB, or Akt) play key roles in the regulation of pancreatic β-cell survival (13–17). The role of the MAP kinase ERK cascade in β-cell survival is controversial. Some studies have suggested an antiapoptotic effect of this cascade (18), whereas others claim that it serves as a mediator of cytokine-induced apoptosis (19–21), this claim in itself being controversial (22). Nevertheless, a possible involvement and impact of PKB/Akt and ERK in outside-in signaling from ECM to β-cells remains to be investigated.

ECM produced by the rat bladder carcinoma cell line 804G is rich in laminin-5 (epiligrin) and also contains fibronectin (23–25). This matrix is able to induce attachment and spreading of many epithelial cell types, including pancreatic β-cells (24,26). Furthermore, our group has shown that this matrix improves β-cell function (27). In the present study, we examined whether the 804G matrix could rescue primary pancreatic β-cells from apoptosis, and we investigated the intracellular pathways involved.

RESEARCH DESIGN AND METHODS

Reagents and antibodies. The following materials were used: PD98059 and LY294002 (Calbiochem, Darmstadt, Germany); In Situ Cell Death Detection Kit (POD; Roche Molecular Biochemicals, Rotkreuz, Switzerland) for terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling

(TUNEL) assay with substrate 3,3′-diaminobenzidine (DAB) from Dako (Carpinteria, CA); cell death detection ELISA^{PLUS} kit (Roche); CaspaTag caspase-8 (LETD) activity kit (Intergen, Oxford, U.K.); Hoechst 33342 (Sigma Fluka, Buchs, Switzerland); propidium iodide (Intergen, Purchase, NY); recombinant rat interleukin-1β (IL-1β; RnD Systems, Abingdon, U.K.) hamster anti-rat CD29 (integrin β1 chain) and hamster IgM control antibodies (Becton Dickinson Biosciences, San Jose, CA); polyclonal anti-phospho-FAK (Tyr-397) and anti-FAK (Biosource International, Camarillo, CA); polyclonal anti-phospho-ERK1/2 (Thr-202/Tyr-204), anti-ERK1/2, anti–phospho-PKB/Akt (Ser-473) and anti-PKB/Akt (Cell Signaling Technology-Bioconcept, Allschwil, Switzerland); monoclonal anti-actin (Chemicon International, Temecula, CA); C-21 anti–inhibitor of nuclear factor (NF)-κBα (IκBα; Santa Cruz Biotechnology, Santa Cruz, CA); anti-mouse horseradish peroxidase (HRP) and anti-rabbit HRP (Amersham Pharmacia Biotech, Dübendorf, Switzerland).

Islet isolation and β-cell purification. All experiments were performed on primary pancreatic β-cells sorted from adult rat islet cells by autofluorescence-activated flow cytometry. Islets of Langerhans were isolated by collagenase digestion of pancreas from male Wistar rats (weighing 150–200 g), followed by Ficoll purification (28). Islets were digested with trypsin and sorted by flow cytometry to obtain β-cells as described (28). This purification procedure yields a population consisting of >95% β-cells (27).

Cell culture. Sorted β-cells were washed and incubated overnight in suspension for recovery as previously described (27). Cells were then resuspended at 4×10^5 cells/ml, and aliquots of 50 μl were plated as droplets on noncoated plastic dishes or on plastic dishes coated with poly-L-lysine (pLL), with 804G matrix, or with laminin-5 purified from 804G (provided by N. Koshikawal and V. Quaranta, San Diego, CA). To assess the effect of the antibody directed against β1 integrin subunit, cells were pretreated for 1 h in suspension with 0.5 μg/ml hamster IgM (control) or with 0.5 μg/ml anti–β1 integrin antibody (Ha2/5) before plating them on pLL- or 804G-coated dishes. When indicated, cells were cultured for 48 h in culture medium containing 1% FCS (serum deprivation). To study the effect of IL-1β, cells were cultured for 48 h in culture medium containing 10% FCS and then treated for 4 h with 2 ng/ml IL-1β. To assess the effects of inhibitors, cells were pretreated for 15 min with 50 μmol/l PD98059 and/or 50 μmol/l LY294002, before plating them on pLL- or 804G-treated dishes. Cells were then cultured in standard medium (10% FCS) for 4 h or for 48 h (fresh inhibitor added at 24 h). DMSO was added to control cells at the same final concentration as that used for the inhibitors. For the protein phosphorylation analysis by Western blot, cells were serum starved for 1–4 h in suspension in serum-free medium containing 0.1% BSA. They were then plated on pLL- or 804G-coated dishes for 30 min in the same medium before performing the protein extraction. For gene expression analysis by low-density gene arrays and by quantitative real-time PCR analysis, cells were serum starved (1% FCS) in suspension for 2 h before plating them on pLL- or 804G-coated dishes (or on noncoated dishes as control for the gene arrays). Cells were then cultured for 18 h in medium containing 1% FCS before mRNA extraction. For the IκBα protein analysis by Western blot, cells were treated as for gene expression analysis and cultured for 24 or 48 h in medium containing 1% FCS before protein extraction. Fresh medium (2 ml) was added every 24 h.

804G matrix preparation. 804G cells (the kind gift of Desmos, San Diego, CA) were grown in Dulbecco's modified Eagle's medium containing 10% FCS and 5.6 mmol/l glucose. Conditioned medium (referred to hereafter as 804G matrix) was prepared as previously described (27).

Coating of plastic dishes with pLL, 804G matrix, and laminin-5. Aliquots (60 μl) of pLL (0.1 mg/ml), crude 804G matrix, or laminin-5 purified from 804G matrix (4 μg/ml in PBS) were layered at the center of 35-mm culture petri dishes (adherent dishes for mammalian cell culture). Dishes were kept in a damp box at 37°C for 18–20 h before being rinsed three times with sterile H_2O and air dried. Uncoated Petri dishes (plastic) or dishes coated with 0.1 mg/ml pLL were used as controls.

Analysis of cell death. To quantify cell death by TUNEL, attached cells were washed with PBS and fixed with 4% paraformaldehyde (20 min at room temperature). After permeabilization with 0.5% Triton X-100 (4 min at room temperature), the TUNEL assay (detecting the free 3-OH strand breaks resulting from DNA degradation) was performed with the In Situ Cell Death Detection Kit, according to the manufacturer's instructions. The preparations were then rinsed with PBS and incubated (10 min at room temperature) with the substrate DAB, and the quantification of dead cells was performed using a light microscope. Unless stated otherwise, results are means ± SD of three independent experiments, with a minimum of 500 β-cells examined for each condition in each experiment. The type of cell death detected by TUNEL was assessed as described by others (29). Cells were stained for 20 min at 37°C with 1 μg/ml Hoechst 33342 (to determine the morphology of the nuclei) and with 1.25 μg/ml propidium iodide (which stains the necrotic nuclei) before fixation and staining with TUNEL reagent. Cells lacking propidium iodide uptake and with condensed nuclei were counted as apoptotic. Late apoptosis (or secondary necrosis) was defined by the uptake of propidium iodide and the presence of nuclear condensation, and necrosis was defined by the uptake of propidium iodide and normal nuclear morphology. Only TUNEL-positive cells were considered for this assessment. The quantification by enzyme-linked immunosorbent assay (ELISA) of mono- and oligonucleosomes present in the cytoplasm of apoptotic cells was performed using the cell death detection ELISA^{PLUS} kit according to the manufacturer's instructions (Roche).

Quantification of caspase-8 activity. To quantify caspase-8 activity, attached cells were treated for 1 h with the fluorescein-labeled peptide FAM-peptide-FMK (which irreversibly binds to active caspase-8) as indicated by the manufacturer (Intergen). At 5 min before the end of the incubation, 25 μmol/l of Hoechst 33342 (final concentration) was added to visualize the nuclei. At the end of the incubation, cells were washed and fixed with 4% paraformaldehyde (20 min at room temperature). The quantification of the number of cells containing active caspase-8 as a percent of total was performed using a fluorescence microscope (Axiocam).

Western blot analysis. To analyze FAK, PKB/Akt, and ERK protein phosphorylation, attached cells were washed with ice-cold PBS without Ca^{+2}/Mg^{+2} supplemented with 1 mmol/l sodium vanadate, and they were lysed in sample buffer 1× (62 mmol/l Tris-Cl, pH6.8, 2% SDS, 5% glycerol, and 1% 2-mercaptoethanol). Protein concentrations were determined with the amido black method (30), and equal amounts of total protein were loaded for SDS-PAGE. To analyze IκBα protein expression, cells were washed with ice-cold PBS and lysed in a buffer containing 150 mmol/l NaCl, 50 mmol/l Tris-HCl, 1% Nonidet P40, 0.25% deoxycholate, 0.1% SDS, 1 mmol/l ditiothreitol, and 1 mmol/l phenylmethylsulfonyl fluoride. After determination of protein concentrations by the method of Lowry, sample buffer 3× was added to the proteins, and the samples were analyzed on an 8% SDS-PAGE gel. All samples, after separation on an SDS-PAGE gel, were electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) for immunoblotting with the appropriate antibody. An enhanced chemiluminescence protein detection kit (Amersham Biosciences) and a Kodak image station were used for visualization of the bands.

Gene expression analysis with microarrays. Low-density membrane cDNA microarrays (Mouse Cancer Pathway Finder GEArray; SuperArray, Bethesda, MD) were used to compare gene expression between cells cultured on plastic and those cultured on 804G matrix. After cell culture, RNA from attached cells was isolated using the QIAshredder and RNeasy Mini Kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. The RNA quality was verified performing agarose gel electrophoresis, and 1.5 μg of the total RNA (for each condition) was used as the template for the ^{32}P-labeled cDNA probe synthesis performed according to the manufacturer's instructions (SuperArray). The membranes were prehybridized and hybridized with the probe, as indicated by the manufacturer, before exposure to X-ray film for various lengths of time at −80°C.

Quantitative real-time PCR. The results for select genes obtained with the microarrays were verified by real-time PCR. cDNA was synthesized with Superscript II (Invitrogen, Basel, Switzerland) using 1 μg of total RNA in a 20-μl reaction volume. For real-time PCR, the cDNA was amplified using a GeneAmp 5700 sequence detection system (PE Applied Biosystems, Foster City, CA). For this purpose, primers were designed according to Primer Express software (PE Applied Biosystems). The following oligonucleotide primer sets were used: IκBα forward: 5′ TGCTGAGGCACTTCTGAAAGC 3′; IκBα reverse: 5′ TCCTCGAAA GTCTCGGAGGTC 3′. The dsDNA-specific dye SYBR Green I was incorporated into the PCR buffer (PE Applied Biosystems) to allow for quantitative detection of the PCR product. The results were analyzed using ABI Prism 7000 SDS software (PE Applied Biosystems). The house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control.

RESULTS

Effect of 804G matrix on β-cell apoptosis induced by serum deprivation or IL-1β. To determine whether the 804G matrix is able to serve as a survival ligand for primary rat pancreatic β-cells, TUNEL and ELISA were performed on cells cultured on 804G matrix or on pLL (a nonspecific adhesive polymer)-coated Petri dishes. β-Cells were cultured for 48 h on pLL-coated (control) or 804G matrix-coated dishes (hereafter called pLL or 804G, respectively) in medium containing either 10% (control conditions) or 1% FCS (low-serum conditions). The number of apoptotic cells as a percent of the total was

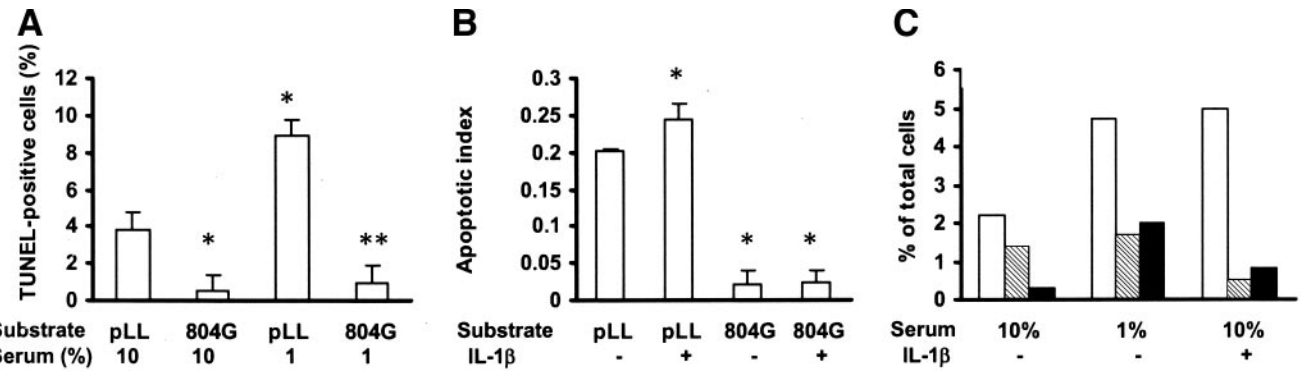

FIG. 1. 804G matrix protects primary pancreatic β-cells from apoptosis. *A*: Cells were cultured for 48 h in medium containing either 10 or 1% FCS before identification of apoptotic (plus necrotic) cells by TUNEL. The number of TUNEL-positive cells as a percent of total was evaluated by optical microscopy. **P* < 0.02 compared with pLL 10% serum; ***P* < 0.001 compared with pLL 1% serum. *B*: After 48 h of culture, cells were treated or not treated with 2 ng/ml IL-1β for 4 h. Apoptosis was measured by ELISA, and apoptotic index is the absorbance [$A_{405\ nm} - A_{490\ nm}$]. Results are means ± SD for *n* = 5 replicates from three independent experiments. **P* < 0.003 relative to pLL (control). *C*: After 48 h of culture and treatment as described above, cells cultured on pLL were stained for 20 min with 1 mg/ml Hoechst 33342 and 1.25 mg/ml propidium iodide for 20 min at 37°C, before fixation and TUNEL analysis. Death was analyzed as described in RESEARCH DESIGN AND METHODS. □, apoptosis; ▨, secondary necrosis; ■ necrosis.

determined under each condition by TUNEL (Fig. 1*A*). Approximately 4% of β-cells cultured on pLL were apoptotic under control conditions. Similar results were obtained when noncoated plastic tissue culture dishes were used instead of pLL-coated dishes (not shown). Culture under low-serum conditions significantly increased the number of TUNEL-positive cells on pLL (9%). When cells were cultured on 804G, <1% of cells were TUNEL positive under control conditions, and serum deprivation did not significantly affect this number.

We next investigated whether the 804G matrix protects β-cells from apoptosis induced by IL-1β, a proinflammatory cytokine that has been shown to be involved in pancreatic β-cell apoptosis leading to the development of type 1 and type 2 diabetes (31,32). For this purpose, the cells were cultured for 48 h on pLL or 804G before being treated for 4 h with 2 ng/ml IL-1β. The apoptosis of β-cells under each condition was measured by ELISA. There was a slight but highly reproducible increase of apoptosis in cells cultured on pLL treated with IL-1β, as compared with nontreated cells ($P < 0.003$). When cells were cultured on 804G, the apoptotic index was significantly decreased as compared with cells cultured on pLL, and there was no difference between nontreated and IL-1β–treated cells (Fig. 1*B*), indicating that this matrix also protects against IL-1β–induced cell death. These results have been confirmed by TUNEL (not shown).

Differential staining of nuclei of TUNEL-positive cells with propidium iodide and Hoechst 33342 distinguished between apoptotic and necrotic cells on pLL (Fig. 1*C*). Under basal conditions (10% FCS), the majority of TUNEL-positive cells were apoptotic or late apoptotic (secondary necrotic). Serum deprivation induced apoptosis and, to a lesser extent, necrosis, whereas IL-1β induced only apoptosis (Fig. 1*C*).

To begin to elucidate the pathway leading to apoptosis on pLL, the activity of caspase-8 in cells cultured for 4 or 48 h on pLL or on 804G was analyzed (Table 1). Caspase-8 activity was higher in cells on pLL compared with those on 804G, and this difference increased with time. These results suggest that the 804G matrix might block caspase-8 activity in turn, explaining the pro-survival effect of the 804G matrix.

Effect of purified laminin-5 on rat islet β-cell survival and spreading. The 804G matrix is known to be rich in laminin-5. However, it also contains fibronectin and may also contain other signaling molecules. To investigate whether laminin-5 is the extracellular molecule responsible for the effect of the 804G matrix, we compared the effects on both cell survival and cell spreading of purified laminin-5 and 804G matrix after 48 h of culture under standard conditions (Figs. 2*A* and *B*). As shown before, there was a significant difference in the number of TUNEL-positive cells between pLL (11%) and 804G (0.5%). The number of TUNEL-positive cells on pure laminin-5 was similar to 804G matrix (Fig. 2*A*). It has been reported that β-cells rapidly flatten and spread on 804G matrix as on pure laminin-5, indicating a strong adhesion of β-cells on these two matrices (27). Here we show that the morphology of β-cells after 48 h of culture on 804G is highly similar to that of cells cultured on pure laminin-5 (Fig. 2*B*). By contrast, rat β-cells do not adhere well and/or do not spread on vitronectin and fibronectin (unpublished results). These results suggest that laminin-5 is the major component of 804G matrix responsible for its pro-survival effect. Therefore, we decided to pursue our studies with the 804G matrix, given its ready availability and convenience of use.

The β1 integrin subunit is involved in the effects of 804G matrix on β-cell survival and spreading. We have previously reported that the laminin-5 receptors integrins α3β1 and α6β1 are expressed on rat β-cells (27,33), and that the latter is involved in the spreading of cells on the 804G matrix (27). To assess whether the β1 integrin subunit is involved in the pro-survival effect of the matrix, we

TABLE 1
Active caspase-8–positive cells

Time	pLL	804G
4 h	2.6 ± 0.9	0.8 ± 0.4*
48 h	4.1 ± 0.5	0.5 ± 0.1†

Data are means ± SD of at least three independent experiments. Rat β-cells were plated on pLL or 804G, and caspase-8 activity was measured after 4 and 48 h. **P* < 0.005 compared with pLL (4 h); †*P* < 0.0002 compared with pLL (48 h).

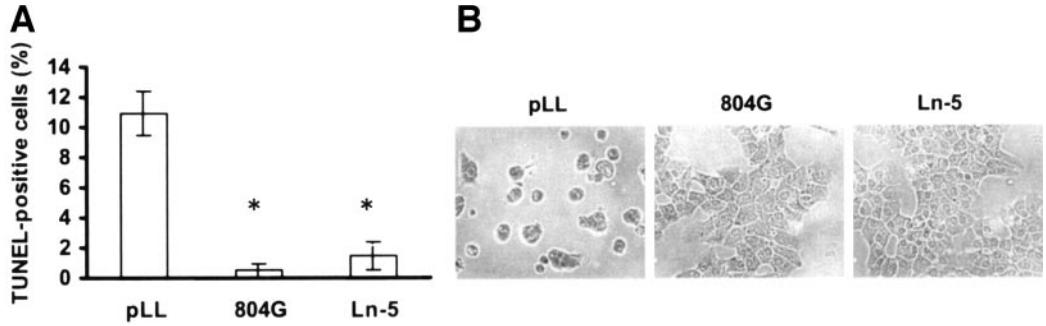

FIG. 2. The spreading and survival of β-cells on purified laminin-5 is similar to that of cells cultured on 804G matrix. *A*: Rat β-cells were cultured for 48 h under standard conditions on pLL-, 804G matrix– or purified laminin-5 (Ln-5)-coated dishes. Apoptotic (plus necrotic cells) were identified by TUNEL. *$P < 0.001$ relative to pLL (control). *B*: Phase-contrast microscopy after 48 h of culture as in panel *A*.

tested the effect of an anti-β1 antibody (Ha2/5) that blocks the adhesion of this integrin to its ECM on the survival of β-cells after 4 h of culture (Fig. 3*A*). On pLL, there was no difference between cells treated with the control antibody IgM and cells treated with Ha2/5. However, on the 804G matrix, the number of TUNEL-positive cells was significantly higher when treated with Ha2/5 (4.9%) compared with control cells (2.3%). Furthermore, the spreading of cells after 4 h of exposure to the 804G matrix was reduced by Ha2/5 (Fig. 3*B*). The control antibody had no effect on cell spreading and cell death at the concentration used (not shown). These results suggest that the β1 integrin subunit mediates the signal from the 804G matrix into the cell, leading to increased spreading and survival.

Effect of 804G matrix on phosphorylation of signaling proteins FAK, PKB/Akt, and ERK. FAK, which is activated by phosphorylation upon integrin ligand binding, mediates survival signaling downstream of integrins and suppresses anoikis and serum withdrawal–induced apoptosis (7,34,35). The PI 3-kinase–PKB/Akt pathway is known to block various apoptotic stimuli. We therefore investigated whether 804G matrix induces activation of FAK and/or PKB/Akt. Cells were serum-starved in suspension for 1 h to reduce constitutive (serum-induced) phosphorylation before plating them for 30 min on pLL or 804G in serum-free medium. Phosphorylation of Tyr397-FAK and Ser473-PKB/Akt was analyzed by Western blot. Adhesion of cells to 804G induced an increased phosphorylation of both proteins compared with adhesion to pLL, whereas the total amount of actin was the same on both substrates (Figs. 4*A* and *B*). These results suggest that this matrix does indeed activate integrin-binding signaling pathways. The amounts of total FAK and total PKB/Akt were slightly decreased in cells on pLL compared with those on 804G (Figs. 4*A* and *B*). It has been reported that caspases cleave specific signaling proteins (including FAK and PKB/Akt) during apoptosis (36). Therefore, the slight decrease of total FAK and PKB/Akt protein amounts in cells cultured on pLL could be due to augmented caspase activity on this substrate compared with matrix.

The FAK signaling complex is known to mediate the activation of the MAP kinase ERK pathway (37,38), a well-known pro-survival pathway in a number of cell types (11). Exposure of serum-starved cells to 804G matrix for 30 min induced higher phosphorylation of ERK1 and ERK2 (Thr-202/Tyr-204 phosphorylation) than pLL (Fig. 4*C*). Attachment of cells to pLL, however, induced higher phosphorylation of ERK proteins as compared with cells in suspension (Fig. 4*C*). In summary, the 804G matrix activates FAK (hallmark of integrin activation) as well as PKB/Akt and ERK pathways.

Signaling pathways involved in pro-survival effects of the 804G matrix. To investigate whether PI 3-kinase–PKB/Akt and ERK pathways are involved in the antiapoptotic effect of 804G matrix, we examined the effects of LY294002 and PD98059. These are specific inhibitors of both PI 3-kinase and MAP kinase/ERK kinase (MEK1), respectively, and were found to be effective in rat β-cells at the concentration used (data not shown). In the short term (4 h), blocking PI 3-kinase and MEK1 activities signifi-

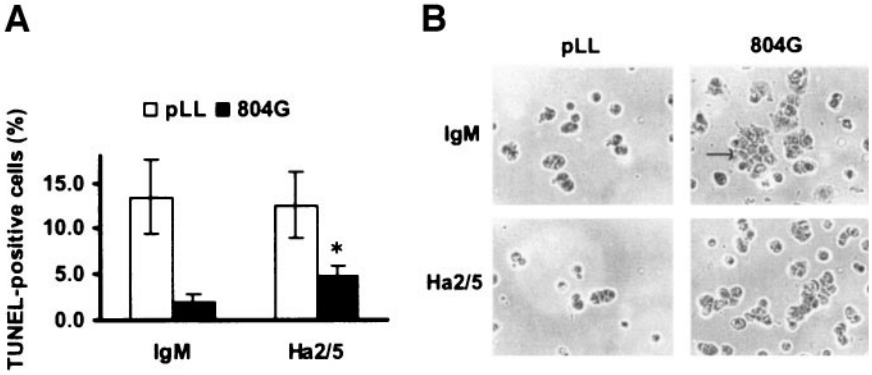

FIG. 3. The β1 integrin subunit is involved in the spreading and improved survival of β-cells induced by the 804G matrix. *A*: Cells were pretreated with control antibody (IgM) or with anti-β1 integrin antibody (Ha2/5) and then attached on pLL- or on 804G-coated dishes. After 4 h of culture under standard conditions, cells were fixed, and cell death was analyzed by TUNEL. *$P < 0.03$ relative to 804G (IgM), pLL (IgM), and pLL (Ha2/5). *B*: Phase-contrast microscopy after 4 h of culture as in panel *A*. A group of spread cells is shown by the arrow.

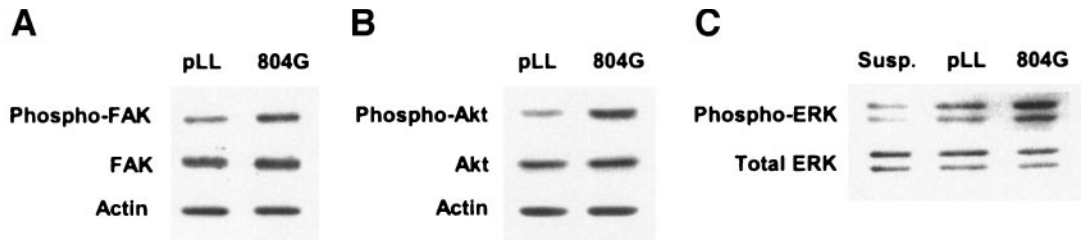

FIG. 4. 804G matrix induces phosphorylation of signaling proteins FAK, PKB/Akt, and ERK. FAK (*A*), PKB/Akt (*B*), and ERK1/2 (*C*) phosphorylation was determined by Western blotting with specific anti-phospho antibodies compared with blotting with antibodies specific for each corresponding protein. Each figure is representative of three independent experiments. Susp., suspension.

cantly augmented cell death on 804G to the same extent (Fig. 5*A*). This treatment did not affect cell death on pLL. The combination of both inhibitors did not alter the effect of each individual inhibitor, suggesting that they block a common pathway activated by the matrix. However, cells on 804G survived better than cells on pLL, even in presence of inhibitors, suggesting that both pathways are only partially involved in the short-term antiapoptotic effect of 804G, or that the inhibitors were only partially effective under these conditions. Blockade of the MAP kinase ERK pathway with PD98059 during 48 h induced a striking increase of the number of apoptotic cells on 804G and also affected cell survival on pLL, albeit to a lesser extent (Fig. 5*B*). In the presence of this inhibitor, attachment to the 804G matrix no longer exerted any protective effect compared with control (pLL). The Western blot analysis (Fig. 4*C*) showed that the attachment on pLL itself induces ERK phosphorylation to a limited extent as compared with suspended cells, although to a lesser extent than on 804G, which may explain why 48 h of treatment with PD98059 also induced higher cell death in cells on pLL. It was also noted that 48 h of culture with PD98059 did not affect the well-spread morphology of the cells cultured on 804G, and that DMSO (used for the dilution of inhibitors) alone had no effect on cell survival and cell spreading at the concentration used (not shown). Treatment of cells with the PI 3-kinase inhibitor LY294002 did not significantly affect cell death on either pLL or 804G after 48 h (Fig. 5*B*), and combined treatment with both inhibitors gave similar results as treatment with PD98059 alone (Fig. 5*B*). These results suggest that the MAP kinase ERK pathway is the dominant one for long-term survival of primary pancreatic β-cells induced by the ECM.

Effect of 804G matrix on gene expression. Differential expression on 804G matrix of genes involved in specific signal transduction pathways such as those implicated in apoptosis and cell cycle was investigated using low-density cDNA microarray membranes (GEArray). Only one gene present on this array was significantly overexpressed in cells cultured for 18 h on the 804G matrix compared with control cells: IκBα (not shown). IκBα has been reported to have an antiapoptotic function in pancreatic β-cells (39). This overexpression was confirmed by real-time quantitative PCR: the IκBα mRNA (normalized to GAPDH mRNA levels) was at least three times more highly expressed in cells cultured on 804G matrix compared with cells cultured on pLL (Fig. 6*A*). IκBα protein levels were measured by Western blot. IκBα protein was increased slightly on 804G vs. pLL after 24 h of culture, with a clear increase by 48 h (Fig. 6*B*). There were no differences in IκBα expression (mRNA and protein) between noncoated and pLL-coated dishes (data not shown). Although this augmented protein expression correlates with the increased gene transcription, we cannot exclude that this difference might be a consequence (partly or completely) of an enhanced degradation of this protein in cells cultured on pLL-coated dishes compared with 804G.

The MAP kinase ERK pathway is involved in IκBα overexpression induced by the 804G matrix. Given that the 804G matrix stimulates the ERK cascade and that this signaling pathway is involved in the pro-survival effects of this matrix, we hypothesized that this cascade might mediate the effect of the matrix on IκBα gene expression. This was tested using the inhibitor PD98059. Treatment of cells with PD98059 did not significantly affect IκBα expression (mRNA) in cells cultured on pLL, but it did reduce by ~60% the IκBα overexpression induced by the 804G matrix (Fig. 6*A*). Thus, these quantitative PCR

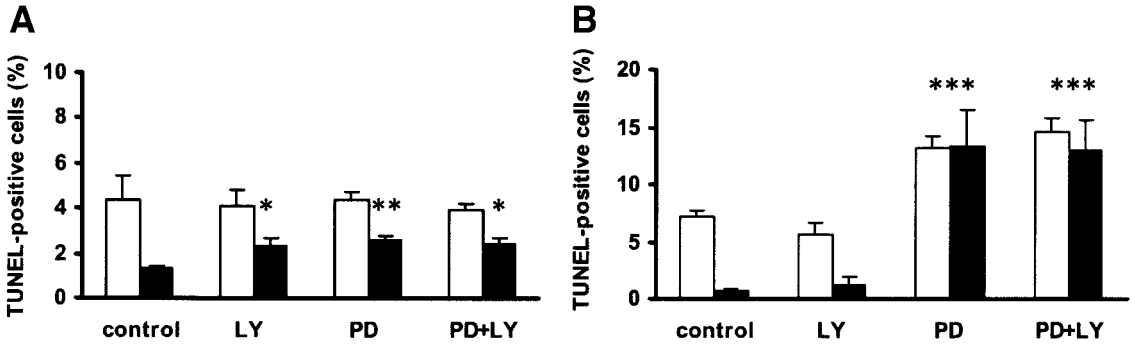

FIG. 5. Involvement of both ERK and Akt/PKB pathways in the pro-survival effect of the matrix. Cells were pretreated with inhibitors of PI 3-kinase (LY294002: LY) and/or of MEK1 (PD98059: PD) and then attached on pLL- or 804G-coated dishes. After 4 h (*A*) or 48 h (*B*), cell death was analyzed by TUNEL. Results are shown as means ± SEM of a minimum of three independent experiments. **P* < 0.04 compared with 804G control; ***P* < 0.01 compared with 804G control; ****P* < 0.005 compared with control conditions (for pLL and 804G). □ pLL; ■, 804G.

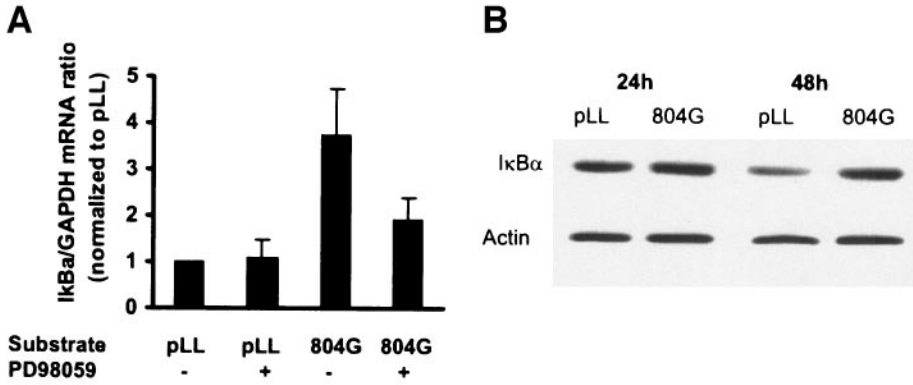

FIG. 6. IκBα expression is induced by the 804G matrix, and this overexpression is reduced by treatment with the ERK inhibitor PD98059. *A*: Cells were treated or not treated with PD98059 before plating them on pLL- versus 804G-coated dishes. After 18 h, RNA was extracted, and real-time PCR was performed. The results (means ± SD from two independent experiments) show the IκBα/GAPDH relative mRNA expression, which was normalized to the control (pLL). *B*: The IκBα protein levels in cells cultured for 24 and 48 h, on pLL or on 804G, were analyzed by Western blotting. This Western blot is representative of three independent experiments.

analyses show that blocking the ERK pathway significantly reduces the effect of the 804G matrix on IκBα gene expression. This result supports the notion that the activation of the MAP kinase ERK cascade by the 804G matrix might contribute to long-term survival of pancreatic β-cells by inducing the expression of antiapoptotic genes.

DISCUSSION

We examined whether ECM can rescue primary pancreatic β-cells from apoptosis and investigated the intracellular pathways involved. The 804G matrix protects pancreatic β-cells from apoptosis under three different conditions: "standard" culture conditions, after serum deprivation, and in response to the cytokine IL-1β. These results demonstrate the importance of adequate ECM for β-cell survival. It is interesting to note that signaling pathways induced by IL-1β in islet β-cells differ when this cytokine is administered in vivo compared with in vitro treatment (40). It has been suggested that one primary difference between the in vivo and the in vitro model systems might be the absence of ECM in the latter (41).

It has been shown that laminin is a component of the basement membrane surrounding islets in vivo, and that the loss of this basement membrane occurring during islet isolation leads to increased cell death (4,42). Experiments performed with laminin-5 purified from 804G matrix (Fig. 2*A* and *B*) suggest that it is the major component of this matrix responsible for its pro-survival effect. The fact that this matrix induces higher phosphorylation of FAK compared with attachment to pLL (Fig. 4*A*) indicates that it engages and activates integrins. Our group has shown that rat pancreatic β-cells express α6β1 and α3β1 integrins (27,33), which have both been identified as receptors for laminin-5. It was shown previously that function-blocking antibodies directed against the α6 integrin subunit blocked cell spreading induced by 804G matrix (27). Here we show that a blocking antibody directed against the β1 integrin subunit reduces both the spreading and the improved survival induced by the 804G matrix (Fig. 3*A* and *B*). Collectively, these data lead us to suggest that α6β1 integrin is a major conduit for outside-in signaling pathway in β-cells established on this particular matrix.

The intracellular molecule(s) responsible for transducing cell-matrix signaling in β-cells have not been studied previously in detail. We now show that compared with pLL, attachment of cells to the 804G matrix induces higher levels of phosphorylation of both MAP kinase ERK and PKB/Akt (Figs. 4*B* and *C*). Furthermore, apoptosis of cells cultured on 804G was increased in the presence of the MEK1 inhibitor PD98059 (4 and 48 h) and/or in the presence of the PI 3-kinase inhibitor LY294002 (4 h only) (Figs. 5*A* and *B*). These results strongly suggest that the short-term pro-survival effect emanating from the 804G matrix is mediated by both of these pathways, whereas longer term effects are mediated at least in part by MAP kinase ERK but not by PKB/Akt.

We propose that the role of both the PI 3-kinase–PKB/Akt and MAP kinase ERK pathways in mediating the short-term (4 h) pro-survival effects of the 804G matrix may be related to their effects on preventing the cleavage and subsequent activation of procaspase-8. Indeed, it has been reported that activated PKB/Akt prevents apoptosis by inhibiting death-inducing signaling complex assembly, thus preventing procaspase-8 cleavage (43), whereas MAP kinase activation deflects DISC signaling from activating caspase-8 (44). This would also explain the nonadditive effect of both inhibitors on short-term cell survival. During the short-term studies, cells on 804G matrix survived better than cells on pLL, even in the presence of inhibitors, suggesting that the ERK and PKB/Akt pathways may be only two components of the short-term antiapoptotic effect of the matrix. A potential mechanism contributing to cell death on pLL, presumably unrelated to ERK and PKB/Akt pathways, is "integrin-mediated death," whereby unligated integrins in cells adhering to substrates devoid of appropriate ECM ligands can act as negative regulators of cell survival (45,46). Unligated integrins are reported to promote apoptosis by recruitment of caspase-8 to the plasma membrane independent of death receptors or Fas-associating protein with a death domain. Our results showing that caspase-8 activation is significantly reduced in cells cultured on 804G compared with cells cultured on pLL (Table 1) support both of the above hypotheses.

The present work shows for the first time the importance of the ERK pathway in promoting survival of pancreatic β-cells induced by the ECM. The fact that the ERK

pathway is involved in the antiapoptotic effect of the 804G matrix on pancreatic β-cells is intriguing because activation of this pathway has been reported to be involved in cytokine-induced apoptosis in β-cells (19,47). One potential explanation for this discrepancy is that cytokine- and ECM-induced activation of ERKs may be significantly different in terms of duration and intensity, which may lead to opposite effects on life-or-death decisions by the cell (48).

The finding that the 804G matrix induces overexpression of IκBα is most interesting because IκBα has been shown to inhibit NF-κB nuclear translocation and transcriptional activity (49). In general, NF-κB is reported to mediate antiapoptotic signals. However, NF-κB is considered as an important transcription factor, mediating IL-1β–induced signal transduction and regulating groups of genes contributing to death in pancreatic β-cells (31). Overexpression of a nondegradable IκB mutant, which specifically blocks cytokine-induced NF-κB activation, prevents β-cell apoptosis (39). The overexpression of the IκBα gene induced by the 804G matrix may thus contribute to its antiapoptotic effect. It is well established that ECM can influence gene expression via the MAP kinase ERK pathway (11). In the present work, we show that activation of the ERK pathway by the 804G matrix mediates the overexpression of IκBα in β-cells. This suggests that activation of the MAP kinase ERK cascade by the 804G matrix might contribute to long-term survival of pancreatic β-cells by inducing the expression of antiapoptotic genes.

Inadequate culture conditions have been suggested to be responsible, at least in part, for the important loss of islet cell viability before transplantation in diabetic patients. A prerequisite for the improvement of islet cell maintenance in vitro, and consequently for increased survival of the grafted cells, is to gain better knowledge of both the intracellular pathways involved in cell survival and factors regulating these signaling pathways. In this context, these results are particularly relevant in providing an insight into important signaling pathways activated by ECM, which lead to improved pancreatic β-cell survival before transplantation.

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