A Novel Approach to Increase Human Islet Cell Mass While Preserving β-Cell Function

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Human islet expansion in monolayer culture leads to loss of function and senescence. By maintaining the 3-D configuration of islets in fibrin gels, it is feasible to expand β-cells in response to hepatocyte growth factor (HGF) while preserving physiologic glucose responsiveness both in vitro and in vivo after transplantation into nude mice. Islets were cultured free floating with or without growth factors and nicotinamide and in fibrin gels with the same conditions. Proliferation was observed only in islets cultured in fibrin gels and the cocktail; total insulin increased by threefold, with a concomitant increase in β-cell mass by morphometry. Insulin release after glucose challenge was also preserved. Islets in fibrin gels gave rise in vivo to large grafts rich in insulin and glucagon, and grafts from free-floating islets were smaller with fewer endocrine cells. Circulating human C-peptide levels were higher than in the mice receiving free-floating islets. In summary, fibrin allows for HGF-mediated cell proliferation while preserving glucose responsiveness in an environment that preserves cell-cell contacts. Limited islet ex vivo expansion under these conditions may improve recipient-donor tissue ratios to equal the functional results of whole-organ transplants. *Diabetes* 51: 3435–3439, 2002

The limited availability of organs precludes the widespread use of islet transplantation in type 1 diabetic recipients. Ex vivo expansion of human islets or alternative sources of tissue for replacement are necessary if cell-based insulin therapies (1) are to compete with whole-organ transplants. Adult human islets replicate after monolayer formation under the influence of hepatocyte growth factor (HGF)/scatter factor (SF) (2) and selected extracellular matrices (3). However, under these conditions, senescence and loss of insulin expression occur (4) with persistence of PDX-1 expression (5). Reaggregation of the expanded cells is required for upregulation of insulin gene expression (6), but the expanded cells are fragile, and removal from the matrix leads to cell death due to both necrosis and apoptosis (7).

By maintaining the islet 3-D configuration while simultaneously providing a fibrin matrix support and inducing β-cell proliferation with HGF/SF, we have found that β-cell mass is augmented, while physiologic glucose responsiveness both in vitro and in vivo after transplantation into nude mice is preserved.

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HGF, hepatocyte growth factor; SF, scatter factor.

RESEARCH DESIGN AND METHODS

Human adult islets. Human adult islets were provided through the Juvenile Diabetes Research Foundation Islet Distribution Program and our islet isolation facility at the University of California at San Diego. They were isolated with an automated method as described previously (8) and further purified by handpicking single islets (100- to 150-μm diameter) after dithizone staining (9).

Islet cell culture. Islets were kept under four tissue culture conditions: free floating in RPMI containing 10% fetal bovine serum and 11 mmol/l glucose with or without a cocktail of growth factors, as well as in fibrin gels with or without the growth factor cocktail. The growth factor cocktail included 20 ng/ml recombinant human HGF (HGF/SF; a generous gift from Genentech), kaposis fibroblast growth factor (hrFGF4, 1 ng/ml; Oncogene Research Products), and 10 mmol/l nicotinamide (Sigma). Fibrin gels were made with human fibrinogen (Sigma) dissolved in PBS (80 mg/ml) and human thrombin (Sigma) dissolved in 40 mmol/l CaCl2 (50 units/ml). After placing the islets in 5-μl droplets of the fibrinogen, an equal volume of the thrombin solution was immediately added to each drop. The liquid polymerized in ~12 min to a soft gel. At that time, medium was added carefully to the dish. Medium and factors were replenished every 48 h.

In vitro cellular growth and survival. Islets in each group were cultured for a total of 6 days. On day 5, after an overnight incubation in RPMI with the glucose concentration reduced to 5 mmol/l, each group was tested for insulin release in response to glucose by incubating at 1.6-mmol/l and 16.7-mmol/l glucose concentrations. On day 6, proliferation rate was assayed by ^{3}H thymidine incorporation as previously described (10). DNA was measured by a fluorometric technique (11). Insulin content and release were measured using a coated antibody radioimmunoassay kit (DPC, Los Angeles) as previously described (10).

In vivo growth and survival. Five hundred islets were cultured alone or with fibrin and growth factors for 1 week, and the resulting cell population was then transplanted under the kidney capsule of athymic nude mice (as previously described) (12) under anesthesia (pentobarbital 50 mg/kg i.p.). This method uses a positive pressure pipette to gently implant the islets under the kidney capsule. Because this was technically difficult to accomplish, the gels were dissolved 24 h before transplantation with streptokinase (3 units/unit thrombin; Sigma). One month later, fasted animals were given 3 g/kg glucose i.p., and after 30 min, blood samples were taken for assay of circulating human C-peptide with a radioimmunoassay assay kit (DPC) that is specific for human C-peptide, with no cross-reaction from mouse C-peptide.

Immunohistochemical analysis and morphometry

In vitro. In some cultures**,** 1 mmol/l BrdU was added to the cultures for 24–72 h before harvesting. Islets were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained. Primary antibodies used were sheep anti-human insulin (The Binding Site), rabbit anti-human glucagon (Chemicon), mouse anti-human cytokeratin 4.62 (CK-19; Sigma), and mouse anti-BrdU (Dako). Secondary antibodies used were rhodamine red–conjugated donkey anti-sheep, fluorescein isothiocyanate–conjugated donkey anti-rabbit, or indodicarbocyanine–conjugated donkey anti-mouse IgGs (Jackson Immunoresearch). Control slides were incubated with a cocktail of the relevant control antibodies (mouse, sheep, and/or rabbit IgGs**).** For morphometric analysis, samples were sectioned completely and all sections examined. β-cell mass was quantified by counting the number of insulin-containing cells per

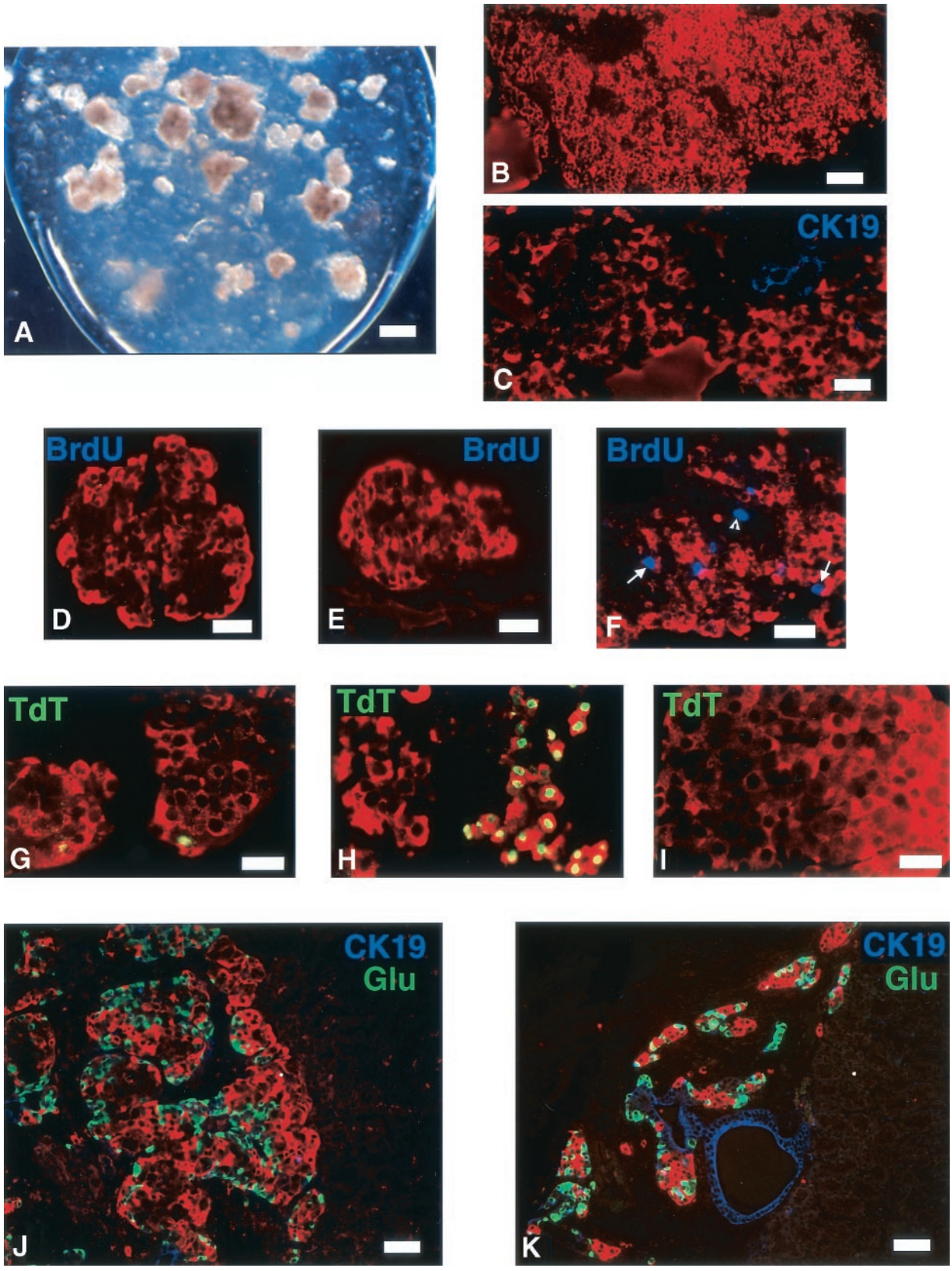

FIG. 1. *A*: Photomicrograph of a fibrin gel containing ~50 islets 2 h after polymerization of a 5-μl fibrinogen solution containing the islets with 5 μl thrombin. *B–I*: Immunohistochemical analysis of islets fixed after 6 days. Most cells in the fibrin gels stain for insulin (red) (*B*). Only rare staining for CK19 (blue) was observed (*C*), indicating that there were very few ductal cells present. Seventy-two hours after addition of BrdU, no labeled nuclei were observed in either free-floating islets with growth factors (*D*) or islets cultured in fibrin in the absence of growth factors (*E*). When the growth factor cocktail was added to the fibrin gels, scattered nuclear labeling (blue) could be seen throughout the gel (*F*). Arrows indicate insulin-containing cells, and the arrowhead indicates non–insulin-containing cells. Apoptotic nuclei were occasionally seen in intact free-floating islets (*G*), and many in disintegrating free-floating islets (*H*), but none in islet cells in fibrin gels (*I*). *J* and *K*: Immunohistochemical

intact islet section. Apoptotic nuclei were detected using the DeadEnd Fluorometric TUNEL system (Promega), which measures the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP(a) at 3′-OH DNA ends using the enzyme terminal deoxynucleotidyl transferase. After secondary staining for insulin, the fluorescein-12-dUTP–labeled DNA was visualized by fluorescence microscopy.

In vivo. Grafts were removed and fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained for insulin, glucagon, and CK-19 as above.

Statistical analysis. Each experimental condition was tested on at least six different preparations of adult islets. Individual in vitro experiments used four replicate cell cultures for each parameter tested. Statistical significance of observed differences was analyzed by ANOVA and Fisher's protected least significance difference test with 95% level as the limit of significance, or Student's t test of paired differences using Statview IV (Abacus Concepts).

RESULTS

Microscopic analysis and morphometry. A few hours after plating in fibrin gels, islets appeared slightly flattened, as observed by phase-contrast microscopy (Fig. 1*A*). After 6 days, immnuohistochemical staining showed that the majority of the cells in the fibrin gel were insulin positive (Fig. 1*B*), and CK19-positive cells were seen only rarely (Fig. 1*C*). BrdU labeling was observed only in the islets that were in fibrin gels and exposed to growth factors (Fig. 1*D*– *F*); in this group, BrdU-positive cells were scattered throughout in insulin-containing (arrows) and non–insulin-containing (arrowhead) cells (Fig. 1*F*), but never in brightly staining insulin-positive cells, as we have previously shown (5). For morphometric analysis, only sections of intact islets were measured; for accuracy, islets that had fused in the fibrin were excluded from analysis. There was a threefold increase in number of β-cells per islet section in fibrin gels treated with growth factors as compared with free-floating islets (100 ± 37 vs. 38 ± 12, $P < 0.0001$). Single apoptotic nuclei were occasionally seen in intact free-floating islets (Fig. 1*G*), and many were seen in disintegrating free-floating islets (Fig. 1*H*), but none were observed in the fibrin-coated islets (Fig. 1*I*).

Fibrin promotes survival and growth. BrdU labeling data were supported by the quantitative assays for thymidine incorporation. After 6 days' culture, proliferation was only observed in the group in which islets were cultured in fibrin gels in the presence of the growth factor cocktail ($n = 4$, $P < 0.0001$) (Fig. 2*A*); in this group, the total DNA also increased threefold ($n = 4$, $P < 0.0001$) (Fig. 2*B*). The islet cells were unable to proliferate in the presence of the growth factor cocktail alone. Although no proliferation was observed in the islets in fibrin without growth factors, as determined both by BrdU (Fig. 1*E*) and ^{3}H thymidine incorporation (Fig. 2*A*), there was a significantly higher total DNA as compared with free-floating islets ($n = 4$, $P < 0.005$) (Fig. 2*B*).

Fibrin increases insulin content. Both insulin/DNA and total insulin were increased significantly after 6 days in fibrin gels ($n = 4$, $P < 0.005$; Fig. 2*C* and $n = 4$, $P < 0.05$; Fig. 2*D*, respectively) as compared with free-floating islets. Thus, the addition of fibrin alone is sufficient to increase insulin content. When the growth factors were added, the total insulin increased threefold over free-floating controls ($n = 4$, $P < 0.0001$) (Fig. 2*D*), which is in agreement with the morphometric data. These findings differ significantly from the situation when cells are grown in monolayer in the presence of growth factors with a concomitant loss of insulin expression (5). The rationale for the growth factors used was based on previous findings: *1*) HGF/SF is mitotic to β-cells (2,6); *2*) fibroblast growth factor-4 has been shown to induce β2/NeuroD in isolated mouse endoderm (13), and, in preliminary experiments, it preserved insulin content in HGF/SF-expanded islet cells (data not shown); and *3*) nicotinamide increases insulin content in β-cells (6,10).

Islets are still functional in fibrin gels. After 5 days in culture, all groups, except for the islets that were free floating in the presence of the growth factor cocktail, were stimulated more than twofold over baseline levels after a glucose challenge (Fig. 2*E*).

Islet function in vivo is improved after culture in fibrin gels. Macroscopically, grafts were larger in mice that had been transplanted with islets grown in fibrin gels than in the same number of islets cultured free floating. This observation was validated by immunostaining (Fig. 1*J*), which showed large compact grafts of endocrine cells (most stained for insulin or glucagon), and CK19 cells were rarely seen. For comparison, grafts from free-floating islets were smaller and showed scattered endocrine cells and also some ductal structures (Fig. 1*K*). After glucose challenge of fasted mice, circulating human C-peptide was significantly higher in mice receiving the graft from fibrin-treated islets than in the mice receiving a comparable number of free-floating cultured islets from the same islet preparation ($n = 6$, $P < 0.005$) (Table 1). In two experiments, we also transplanted islets within 24 h of isolation. Higher C-peptide levels were also observed when grafts from fibrin-treated islets were compared with grafts of freshly isolated islets from the same preparation transplanted without the 7-day culture period (data not shown).

DISCUSSION

3-D matrices such as collagen gel have previously been shown to promote survival and differentiation of islets in culture (14–19), but neither their effect on proliferation nor the mechanisms responsible for their beneficial effect have been studied. In this report, we show that a 3-D fibrin matrix support improves survival, reduces apoptosis, and allows for HGF-mediated proliferation of islet cells, leading to increased β-cell mass without loss of function.

Previous data have shown that islet proliferation needs the interaction in monolayer with matrices such as 804G or HTB-9 (2,3). Our findings in this report show that although growth factors are ineffective in islets that are free floating, cellular interaction in a 3-D configuration with a fibrin matrix allows proliferation to occur. Thus, avoiding cell growth in monolayer eliminates the need to disrupt cell matrix interactions and subsequent anoikis (7). Moreover, we show that glucose-responsive β-cell function is not impaired in proliferating islet cells in fibrin, which is in contrast to the loss of function seen previously

analysis of islet grafts 1 month after transplantation. *J*: Islets that had been previously cultured in fibrin gels; large grafts were full of compact clusters of endocrine cells, mostly insulin (red) and some glucagon (green). Only rare staining for CK-19 (blue) was seen. For comparison, islets cultured free floating gave rise to smaller grafts, with scattered endocrine cells (*K*). In some areas, ductal structures (blue CK-19 staining) could be seen. Bar = 120 μm in *A*; 60 μm in *B*, *J*, and *K*; 30 μm in *C*– *F*; and 15 μm in *G*– *I*.

TABLE 1
Comparison of human C-peptide levels in mice transplanted with islets previously cultured free floating or in fibrin

Islet preparation	Control grafts serum C-peptide (pmol/l)	Fibrin grafts serum C-peptide (pmol/l)	Increase over control
1	184	457	2.5
2	147	588	4
3	327	648	2.0
4	204	347	1.7
5	938	1,644	1.8
6	674	1,134	1.7

One month after transplantation, mice were fasted overnight and then challenged with 3 g/kg glucose i.p. After 30 min, blood was drawn from the external jugular for assay of serum human C-peptide, using a radioimmunoassay kit that does not cross-react with mouse C-peptide ($n = 6, P < 0.005$ for paired comparison of islets from each preparation).

in islets expanded in monolayer on matrices such as HTB-9 (5).

We and others (20,21) have shown that loss of islet cell mass through apoptosis occurs rapidly after islet isolation. The higher total insulin and DNA contents observed in the islets in fibrin without growth factors, as compared with free-floating islets, was concomitant with the lack of cell proliferation, as determined both by BrdU and ^{3}H thymidine incorporation and the absence of apoptotic nuclei, and could be indicative of increased cell survival and/or suppression of cell death mediated by β-cell fibrin-serum interactions.

Fibrin gels have been shown to support cell survival, growth, and differentiation through the intermediary function of specific integrins, including α5β1, αvβ3, and αvβ1 (22–24) Based on preliminary studies using function-blocking antibodies, we have identified αvβ1 as an important candidate in the adhesion of adult β-cells to purified fibrin (data not shown). However, further studies are

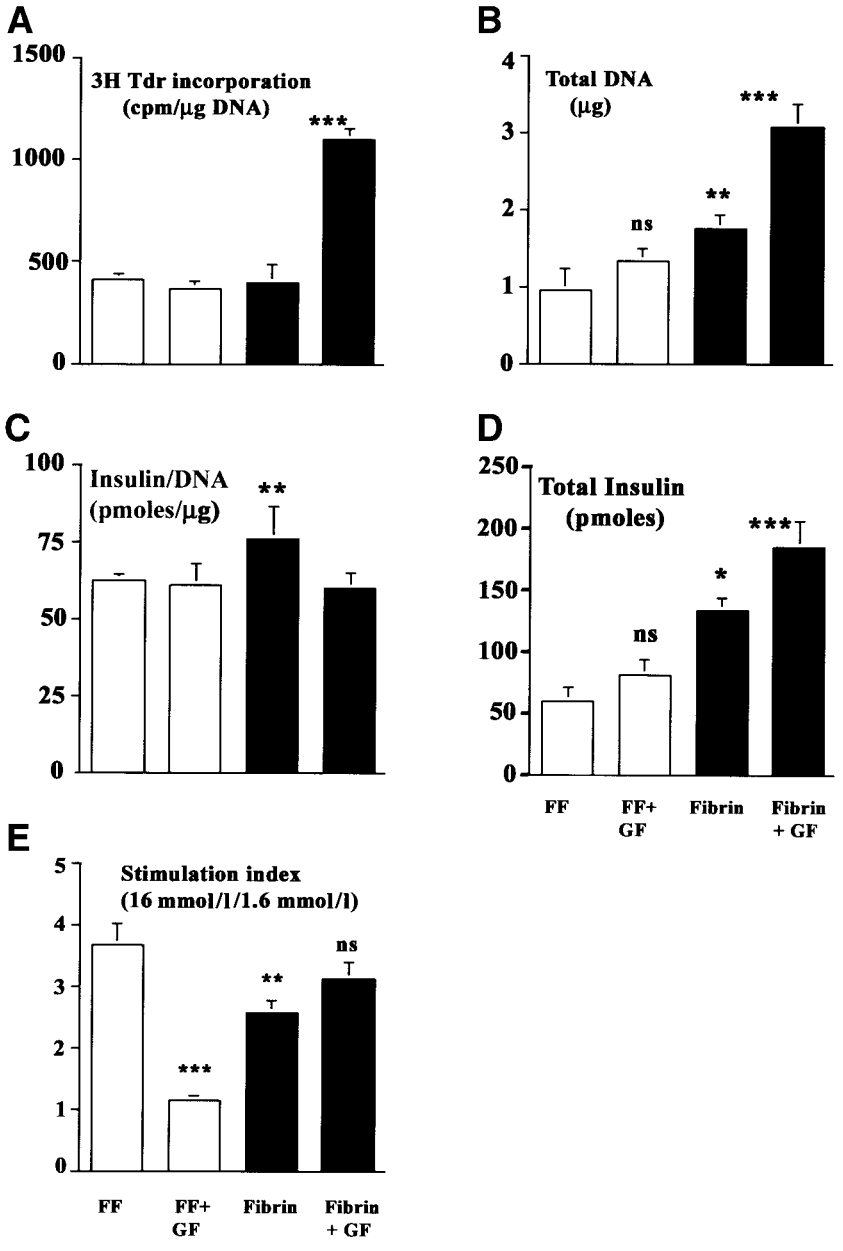

FIG. 2. Representative experiment of quantitation of growth (*A* and *B*), insulin content (*C* and *D*), and β-cell function (*E*) of islets cultured for 6 days free floating or in fibrin gels with or without the growth factor cocktail. For each parameter tested: $n = 4$; *$P < 0.05$, **$P < 0.005$, ***$P < 0.0001$ compared with free-floating islets, showing that fibrin increases both total DNA and insulin content, that with the addition of growth factors proliferation is induced, and that fibrin does not impair the ability to respond to a glucose challenge.

required to identify whether this integrin or others are ultimately responsible for supporting long-term β-cell survival or proliferation in fibrin gels supplemented with growth factors and serum.

The results of the islet transplants show that previous culture in fibrin gels ensures a robust graft that is capable of withstanding the deleterious conditions of engraftment. After glucose challenge, mice with grafts from fibrin-treated islets responded with higher C-peptide levels than mice with islets transplanted either within 24 h after isolation (as in the Edmonton protocol) or after 1 week in culture. Thus, the improvement in islet survival and function was due to the previous culture period in fibrin, and graft survival and function could perhaps be further improved by transplantation of the islets in fibrin, given that fibrin is also known to promote angiogenesis (25,26). However, dissolution of the gel by substances such as streptokinase, as described here, would facilitate the clinical use of islets for intraportal delivery.

In summary, fibrin improves survival of β-cells in vitro and reduces apoptosis, and addition of growth factors promotes proliferation and increased β-cell mass without loss of function. Thus, limited ex vivo islet expansion may improve recipient/donor tissue ratios to equal the functional results of whole-organ pancreas transplants.

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