

Intracellular Stress Signaling Pathways Activated During Human Islet Preparation and Following Acute Cytokine Exposure

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Pancreatic islet transplantation may successfully restore normoglycemia in type 1 diabetic patients. However, successful grafting requires transplantation of a sufficient number of islets, usually requiring two or more donors. During the isolation process and following clinical transplantation, islets are subjected to severe adverse conditions that impair survival and ultimately contribute to graft failure. Here, we have mapped the major intracellular stress-signaling pathways that may mediate human islet loss during isolation and following cytokine attack. We found that the isolation procedure potently recruits two pathways consisting of [mitogen-activated protein kinase (MKK)7 → Jun NH₂-terminal kinase (JNK)/p38 → c-fos] and the [nuclear factor-κB (NF-κB) → iNOS] module. Cytokines activate the [NF-κB → iNOS] and [MKK4/MKK3/6 → JNK/p38] pathways without recruitment of c-fos. Culturing the islets for 48 h after isolation allows for the activated pathways to return to background levels, with expression of MKK7 becoming undetectable. These data indicate that isolation and cytokines recruit different death pathways. Therefore, strategies might be rationally developed to avoid possible synergistic activation of these pathways in mediating islet loss during isolation and following grafting. *Diabetes* 53:2815–2823, 2004

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Received for publication 6 February 2004 and accepted in revised form 30 July 2004.

DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; IFN γ , γ -interferon; IκB, inhibitor of κB; IL-1 β , interleukin-1 β ; iNOS, inducible nitric oxide synthase; JNK, c-Jun NH₂-terminal kinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; NF-κB, nuclear factor-κB; PNF, primary graft nonfunction; TLM, two-layer cold storage method; TNF α , tumor necrosis factor- α .

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Type 1 diabetes is an autoimmune disease resulting from a progressive decrease in β -cell mass and function (1). The mediators of β -cell dysfunction and destruction include macrophages, T-cells and their inflammatory products, such as cytokines (2), and free radicals such as nitric oxide (NO) (3–5). Pancreatic islet transplantation is one strategy that holds promise to cure patients with type 1 diabetes (6), but purified pancreatic islet grafts have failed to maintain long-term glucose homeostasis in human recipients; the reasons for this failure are still poorly understood (7,8). There is however a more immediate problem with islet grafting that is dependent on poor islet recovery from donors (9) and early islet loss following the first hours of grafting (10,11). This tendency of islet grafts to fail to function within a short period after transplantation is termed primary graft nonfunction (PNF). Indeed, the islet isolation procedure itself destroys cellular and noncellular components of the pancreas that probably play a role in supporting islet survival (10,12). Further islet transplantation exposes cells to a variety of stressful stimuli, notably proinflammatory cytokines that encourage β -cell death and lead to early graft failure (13–15). Regardless of the molecular mechanisms involved, a reduction in islet β -cell mass after transplantation implicates islet β -cell death by apoptosis and the prerecruitment of intracellular death-signaling pathways (16,17).

Death-signaling pathways that have been shown to contribute to β -cell death in vitro include the transcription factor nuclear factor-κB (NF-κB) (17) and the stress group of the mitogen-activated protein kinases (MAPKs) (18,19), and pharmacological inhibition of these pathways has proven beneficial in different models of insulin-secreting cell death in vitro (20,21). NF-κB is a ubiquitously expressed transcription factor that regulates a family of inducible genes (22). At rest, NF-κB is found in the cytosol in an inactive form bound to the inhibitory protein inhibitor of κB (IκB) α (23). Cytokines and other stresses induce the phosphorylation of IκB α , which then undergoes degradation, releasing NF-κB that translocates into the nucleus (24). One of the major genes upregulated by NF-κB in rodent islets and that contributes to apoptosis is the inducible NO synthase (iNOS) gene. Regulation and activation of MAPKs appear to require more signaling events.

MAPK core module pathways are composed of three conserved kinases that proceed through a phosphorylation cascade, creating a sequential-activation pathway (25). The first kinase in the module is an MAPK kinase kinase, a serine/threonine kinase that, when activated, phosphorylates and activates the next kinase in the module, an MAPK kinase (MKK). The MKKs are dual-specific kinases that phosphorylate a Thr-X-Tyr motif in the activation loop of MAPKs. MAPKs are the final kinases in the module and phosphorylate different substrates on serine and threonine residues (26,27). Three major conserved groups of MAPKs have been described: the extracellular signal-regulated kinases (ERKs; ERK1/2/3) (28), the p38 kinases (p38 $\alpha/\beta/\gamma/\delta$) (29), and the c-Jun NH₂-terminal kinases (JNKs; JNK1/2/3) (30). In mammals, these MAPKs can be activated by six MKKs: MKK1 and -2 are upstream activators of ERK1/2 (31), MKK3 and -6 are activators of p38 (32–34), and MKK4 and -7 regulate JNK (35,36). It has also been shown that MKK4 can activate p38 in addition to JNK (37). Multiple stresses, including ultraviolet and γ irradiation, cytotoxic drugs, cold and heat shocks, loss of survival factors, hypo- and hyperosmolarity, proinflammatory cytokines, shearing stresses, and reactive oxygen species (38,39), can similarly lead to both JNK and p38 activation, hence the name “stress kinases.” Both JNK and p38 activate downstream nuclear transcription factors that participate to the cellular response (40,41). Among these, activation of the activator protein-1, which is formed of heteromers of c-Fos, c-Jun, and ATF2, is required for some forms of apoptosis, notably in neuronal cells (42,43). A major transcriptional target of JNK and p38 is the *c-fos* gene.

During the isolation procedure and immediately following grafting, human islets are exposed to diverse stressful stimuli that lead to islet damage. The goal of this study is to provide a map of the recruitment of the aforementioned stress-signaling pathways that could participate in the dysfunction and destruction of human islets during isolation and following acute cytokine attack.

RESEARCH DESIGN AND METHODS

Human islet isolation. The abdominal organs from a cadaveric donor are perfused with University of Wisconsin preservation solution at 4°C and the pancreas removed. The current techniques for isolating islets require that pancreata stored in cold University of Wisconsin solution be processed within 12 h in order to prevent islet deterioration. Additional storage using the two-layer cold storage method (TLM; “pancreas” step), which uses University of Wisconsin solution and perfluorochemical, extends the acceptable preservation period of pancreata before islet isolation and increases islet yields. Thus, after the pancreas is trimmed of extra tissue and the main pancreatic duct cannulated, the pancreas is stored at 8°C for an additional 3 h on TLM. Islet isolation is performed as described in the method of Ricordi et al. (44), which combines enzymatic digestion and mechanical dissociation. Briefly, after injection of an enzyme solution (Liberase-HI; Roche Molecular Biochemical, Indianapolis, IN) into the main pancreatic duct, the distended pancreas is cut into several pieces and put in a temperature-controlled chamber (“digestion” step) that allows progressive heating up to 37°C. To allow for better pancreas fragmentation, the chamber is agitated using the hand-shaking technique. The digested pancreas is collected by centrifugation and washed (“washing” step) before density gradient purification. At that stage, the preparation contains fragmented exocrine tissue with 1–2% free intact islets. The tissue suspension is then purified over a continuous-density gradient using a COBE 2991 cell processor (“purification” step). The islet number is determined following dithizone staining and is expressed as the number of islet equivalents (1 islet equivalent = 150- μ m diameter islet). The purity of the human islet preparations used in this study was >70%. The human subjects exemption code from the institutional review board is 0307M50821.

Human islet culture. Purified human islets are kept in culture in CMRL-1066 medium (Mediatech, Herndon, VA) supplemented with 10% heat inactivated FBS (Mediatech) for a period of 2 days (37°C for 12–24 h and 28°C for 24–36 h) in a 5% CO₂-humidified atmosphere. Cultured islets are treated for 45 min with recombinant human interleukin-1 β (IL-1 β) alone or in combination with recombinant human tumor necrosis factor- α (TNF α) and recombinant human γ -interferon (IFN γ) (each used at 10 ng/ml). All cytokines were purchased from R&D systems (Minneapolis, MN).

Islet cell lysis and Western blotting experiments. Human islets are washed once in cold PBS and recovered by centrifugation. Briefly, cell pellets are dislodged into cold lysis buffer (20 mmol/l Tris-acetate, pH 7.0, 0.27 mol/l sucrose, 1 mmol/l EDTA, 1 mmol/l EGTA, 50 mmol/l sodium fluoride, 1% Triton X-100, 10 mmol/l β -glycerophosphate, 1 mmol/l dithiothreitol [DTT], 10 mmol/l *p*-nitrophenylphosphate, and antiproteases) and the cells sonicated for 20 s and centrifuged at 15,000*g* for 20 min. The supernatants are recovered and stored at –70°C until use. Total protein in cell lysates is quantified by the Bradford method (Bio-Rad Bioassay Kit). Equal quantity of total protein lysates are resolved by 4–20% gel SDS-PAGE (Invitrogen) and electroblotted onto polyvinylidene fluoride membranes. The blots are then probed with primary antibodies for MKK7, MKK4 (1/2,000; Upstate), phospho-MKK4, MKK3, phospho-MKK3/6, p38, phospho-p38, I κ B α (1/1,000; Cell Signaling), and tubulin (1/1,000, Santa Cruz). The secondary antibodies are used at 1/3,500 (Bio-Rad). Immunoblots are visualized using an enhanced chemiluminescence detection system (Amersham Life Sciences).

Protein kinase assays and gel quantification. Islet protein extracts (40 μ g) are incubated for 3 h at 4°C with 1 μ g of the fusion proteins glutathione S-transferase (GST)-c-Jun and GST-Elk1 coupled with glutathione beads. After centrifugation (2,000*g* for 2 min), the supernatant is removed and the beads washed twice. The pellets are then resuspended in 20 μ l kinase buffer (20 mmol/l HEPES, pH 7.5, 20 mmol/l β -glycero-phosphate, 10 mmol/l MgCl₂, and 1 mmol/l DTT) and incubated with 1 μ l [³²P] ATP (3,000 Ci/mmol) (Amersham) at 30°C for 30 min. The reactions are terminated by addition of 1 \times Laemmli sample buffer (50 mmol/l Tris HCl, pH 6.8, 2% SDS, 100 mmol/l DTT, 0.1% bromophenol blue, and 10% glycerol). Phosphorylation of substrate proteins is examined after SDS-PAGE (Bio-Rad). The gels are exposed overnight to autoradiography and quantified by Phosphor-Imager analysis.

Measure of mRNA expression by real-time RT-PCR. Total RNA is isolated from human pancreata using a commercial kit (RNeasy Mini-kit protocol; Qiagen) and quantified by spectrophotometry. Two micrograms of RNA are reverse transcribed to cDNA (1 h at 37°C and 5 min at 95°C) using random primers (Gibco-BRL). We applied a quantitative real-time RT-PCR technique to measure gene expression. This method has been described in detail (see manufacturer literature: Light Cycler; Roche Molecular Biochemicals). The PCR uses the following primers: c-Fos: sense 5'-TGATACACTCCAAGCGGA GAC-3' and antisense 5'-CCCAGTCTGCTGCATAGAAGG-3'; insulin: sense 5'-GTGCGGGGAACGAGGCTTCTTC-3' and antisense 5'-GCGGCGGGTGTGG GGCTGCC-3'; iNOS: sense 5'-ACGTGCGTTACTCCACCAACA-3' and antisense 5'-CATAGCGGATGAGCTGAGCA-3'; and finally the housekeeping gene β -actin: sense 5'-AACGGCTCCGGCATGTGCAA-3' and antisense 5'-AGTACA TCGCTGTGCACGTGGT-3'.

Aliquots of the generated cDNAs (200 ng) are loaded into capillary tubes and amplified for 40 cycles. The PCR products are further analyzed by agarose gel electrophoresis to confirm the correct length of the amplified products.

Electrophoretic mobility shift assays. The assays are performed using two annealed complementary synthetic oligonucleotides for NF- κ B binding (purchased from Promega): NF- κ B: sense 5'-AGCTAGCTTCAGAGGGGACTTTCC GAGAGG-3' and antisense 5'-AGTCTCTCTCGGAAAGTCCCTCTGAAGCT-3'. The double-stranded oligonucleotide (4 pmol) is end labeled with [³²P] ATP (3,000 Ci/mmol) (Amersham). Nuclear protein extracts are prepared from human purified islets that were lysed as described above for kinase assays. Following centrifugation, the nuclear pellets are resuspended in 100 μ l high-salt buffer (20 mmol/l HEPES, pH 7.9, 1 mmol/l EDTA, 1 mmol/l MgCl₂, 10 mmol/l KCl, 20% glycerol, 1 mmol/l DTT, 400 mmol/l NaCl, and antiproteases). Protein concentration is determined by the Bradford method. For EMSAs, 10 μ g nuclear extract are incubated for 30 min on ice with the labeled probe in EMSA binding buffer (100 mmol/l HEPES, pH 7.9, 50 mmol/l NaCl, 5 mmol/l MgCl₂, 5 mmol/l EDTA, 50% glycerol, and 5 mmol/l DTT). Poly (dI-dC) (1 μ g/ μ l) is used as an unspecific DNA competitor in each reaction. Specific and unspecific competitor oligonucleotides are incubated with the nuclear extracts on ice for 15 min before addition of the probe. DNA-protein complexes are resolved on a 6% nondenaturing polyacrylamide gel in 1 \times Tris-borate-EDTA buffer (44 mmol/l Tris-HCl, pH 8.0, 44 mmol/l boric acid, and 1 mmol/l EDTA, pH 8.0). Gels are fixed for 10 min (10% methanol and 10% acetic acid) and dried for 40 min at 80°C. NF- κ B DNA binding activity is visualized by autoradiography.

TUNEL assays. Isolated human islets were washed in PBS, collected by centrifugation, and added to 2% agarose gel. Cryostat sections are then used for TUNEL assay according to the manufacturer's instructions (In Situ Cell Death Detection Kit, POD; Roche Boehringer Mannheim). The kit uses an anti-fluorescein antibody conjugated to peroxidase.

Statistics. We analyzed pancreas and islet specimens from nine different deceased human multiorgan donors. In each case, the purity of human islet preparations was between 66 and 70%. All experiments were performed a minimum of three times (i.e., with three different donors) in duplicate. For some experiments, all nine donors were examined (i.e., $n = 3-9$ for all data). All results are presented as a means \pm SD. Statistical analysis for multiple comparisons was determined by one-way ANOVA (post hoc comparisons, Scheffé test). The differences found between the experimental groups were considered statistically significant at $P < 0.05$ or $P < 0.01$.

RESULTS

MAPK level: activity of JNK, p38, and ERK during islet isolation and in response to cytokines. We first measured the activities of the three MAPKs JNK, p38, and ERK at each main step of the islet isolation process and following cytokine treatment. Samples were collected from pancreas following tissue digestion, after the washing step, and at the end of the procedure with purified islets. Cultured islets were also incubated with or without cytokines, before protein and RNA extraction.

Activity of JNK was low in pancreas and became progressively higher during the isolation procedure. JNK activity in total pancreatic extracts (>98% exocrine tissues) increased markedly (fivefold) following digestion and after the wash step (sevenfold) (Fig. 1). This high level of activity persisted up to the end of the isolation procedure (purified islets). JNK activity then declined to a low baseline level over 2 days of culture. Short-term exposure of the cultured islets to the cytokine IL-1 β alone increased JNK activity to the same high level as during the isolation process. Addition of the three cytokines in combination (IL-1 β , TNF α , and IFN γ) did not enhance JNK activity further than IL-1 β alone (data not shown).

p38 activity was already high in pancreas and persisted throughout the procedure up to the actual islet purification. p38 activity decreased after 48 h of culture. In contrast to JNK, p38 did not appear to respond markedly to IL-1 β stimulation, but nevertheless, activation appeared significant (Fig. 1). Finally, ERK activity was relatively unaffected throughout the isolation procedure (Fig. 1). ERK is not a bona fide stress pathway (28,39); however, it provides an internal control in these experiments.

Collectively, these data indicate that the isolation process is as potent in activating JNK as IL-1 β alone or the mix of cytokines IL-1 β , TNF α , and IFN γ . However, the procedure appears to be a more potent inducer of p38 activity than cytokines. It is remarkable to note that the isolation procedure leads to a sustained activation of the two stress kinases throughout all steps of isolation, lasting well over several hours. This certainly has profound implication for cell survival and function (45).

Downstream of MAPKs: induction of *c-fos* mRNA.

C-fos expression is under the control of a wide variety of stimuli and many signaling pathways, with the three MAPKs probably playing a dominant role in many cell types. In β -cell lines, *c-fos* is under the direct control of JNK, whose activation appears necessary for its expression (20). Increased *c-fos* mRNA expression was detected in digested and washed tissues, with maximal levels reached at the end of purification (Fig. 2). After 2 days of

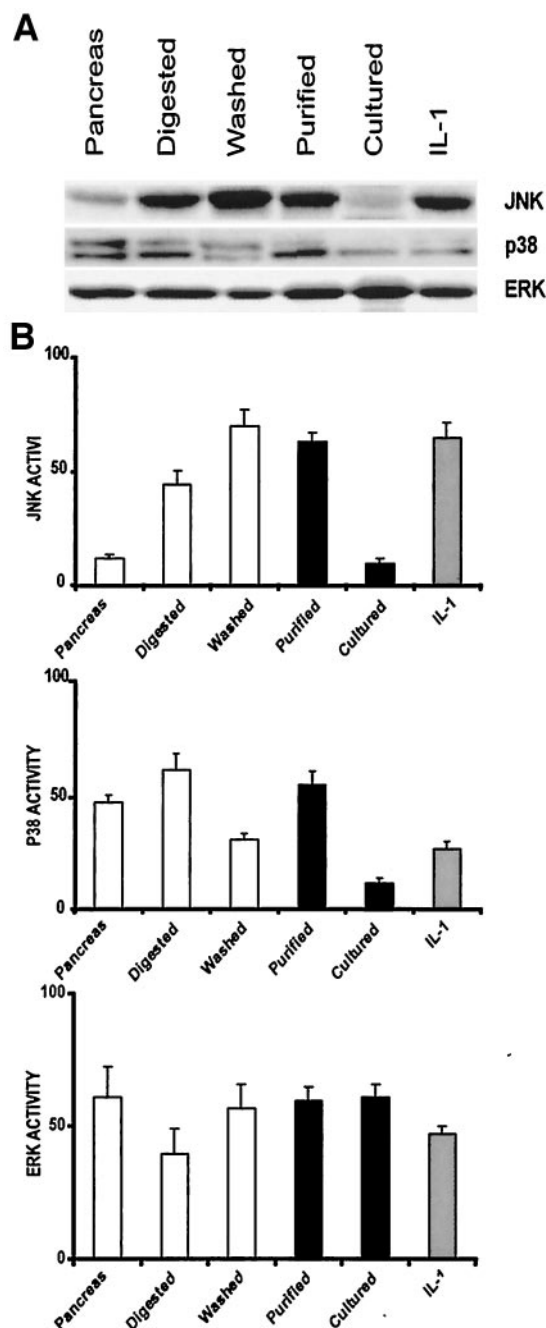


FIG. 1. Effect of the islet isolation procedure and IL-1 β stimulation on activation of JNK, ERK, and p38. **A:** Representative experiments. For measuring JNK and ERK activities, we used GST-Jun (JNK) and GST-Elk1 (ERK) as substrates, as described in RESEARCH DESIGN AND METHODS. Photographs represent $\gamma^{32}\text{P}$ -phosphorylation of the substrates (P-GSTs) following SDS-PAGE. The level of p38 phosphorylation was determined by immunoblotting using an anti-P-p38 antibody, and p38 levels were estimated using an anti-p38 antibody for normalization. **B:** Quantification of data in A (arbitrary units). JNK activity: $n = 5$, $P < 0.01$ for purified versus cultured islets; $P < 0.05$ for IL-1 β (IL-1)-treated versus cultured islets. Differences are also significant with $P < 0.01$ for all data compared with TLM. p38 activity: $n = 5$, $P < 0.01$ for digested, washed, and purified preparations versus cultured islets; $P < 0.05$ between IL-1 β -treated versus cultured islets. ERK activity: $n = 5$, no significant difference was found among all groups. □, total pancreatic tissues (mainly exocrine, >98%); ■, purified endocrine tissues (>70%); ▨, purified islets incubated with cytokines.

culture, *c-fos* returned to a low basal level. Surprisingly, acute cytokine treatment (45 min) did not induce *c-fos* expression despite a marked activation of JNK and a slight

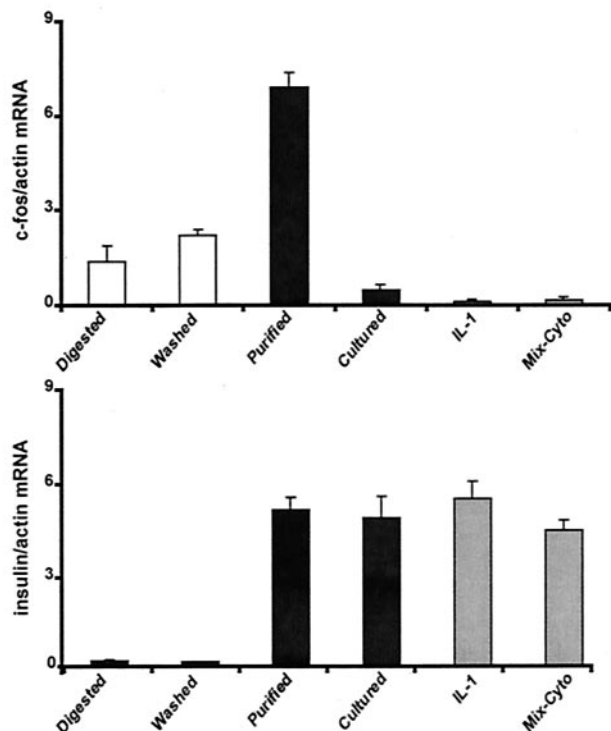


FIG. 2. Effect of islet preparation and cytokines stimulation on the expression of the *c-fos* and insulin mRNAs. Human islets samples were taken after each main step of islets isolation (pancreas, digestion, wash, and purification). After culture (48 h), the cells were exposed for 45 min to IL-1 β (10 ng/ml) alone or in combination with the cytokines TNF α and IFN γ (10 ng/ml). Total RNAs were extracted and RT-PCRs were performed as described in RESEARCH DESIGN AND METHODS. Quantification of *c-fos* (top) and insulin gene expression (bottom) by real-time RT-PCR (LightCycler). The β -actin mRNA was used for normalization. $n = 7$, $P < 0.01$ when comparing *c-fos* mRNA expression in cultured versus purified islets. No statistical differences were observed between the cultured and IL-1 β (IL-1)- and cyto-mix (Mix-Cyto)-treated groups. \square , total pancreatic tissues (mainly exocrine, >98%); \blacksquare , purified endocrine tissues (>70%); \blacksquare , purified islets incubated with cytokines.

activation of p38 in these conditions. As controls, neither insulin nor actin mRNA levels appeared to be markedly affected by these treatments (Fig. 2).

Upstream of JNK and p38: activation and recruitment of MKK4, MKK7, and MKK3/6. Two JNK kinases (i.e., kinases able to activate JNK) have been described: MKK4 and -7. Biochemical studies indicated that in different cell types, MKK7 is responsible for activation of JNK by cytokines, whereas MKK4 responds to other stimuli like irradiation, osmotic shocks, etc. MKK4 might also activate p38, although this last activity is controversial (C. Widmann, personal communication).

To determine which upstream pathways lead to JNK activation during the isolation procedure versus cytokine attack, we first determined the level of MKK7 expression by Western blotting of the same extracts that we used for measuring JNK activity (Fig. 1A). MKK7 appears equally abundant in exocrine tissues and in purified islets immediately following the isolation procedure (Fig. 3A). Remarkably, MKK7 expression declined following islet culture, reaching undetectable levels after 48 h. Neither IL-1 β nor the mix of cytokines (data not shown) appeared to restore MKK7 expression. Due to the unavailability of antibodies specifically recognizing the activated form of

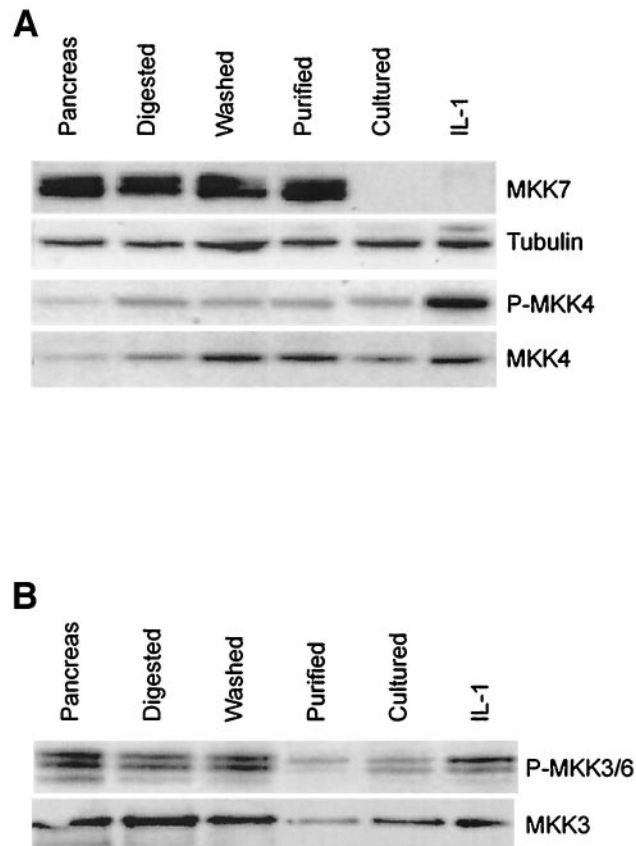


FIG. 3. Expression profile and activation of the upstream JNK kinases MKK7 and -4 and of the upstream p38 kinases MKK3/6 during the islet isolation procedure. Samples were taken during the main steps (pancreas, digestion, wash, and purification) of the isolation procedure and then cultured for 48 h and treated with IL-1 β for 45 min before Western blot analysis. (A). The blots were probed with anti-MKK7 and anti-P-MKK4. Results show the expression level of the MKK7 protein before and after purification of islets, after islet culture, and following IL-1 β stimulation. Equal protein loading was assessed by blotting membranes with an antibody against tubulin. Blots were also probed with an anti-P-MKK4 antibody to measure the activation of MKK4. The level of total MKK4 (phosphorylated or not phosphorylated) was also determined by Western blotting. Data represent the mean of four independent experiments performed in duplicate. (B): Total lysates from human pancreata taken at the indicated steps were subjected to Western blot analyses using a polyclonal antibody recognizing both the phosphorylated forms of MKK3 (Ser¹⁸⁹Thr¹⁹³) and MKK6 (Ser²⁰⁷Thr²¹¹) simultaneously. Immunoblotting with an anti-MKK3 antibody was performed for normalization. Results are representative of three independent experiments.

MKK7 (phospho-MKK7), we were unable to correlate the presence of MKK7 with its activation by the isolation procedure. Nevertheless, these data indicate that whereas MKK7 might transduce stress signaling to JNK during human islet isolation, it certainly does not follow cytokine attack, as the kinase does not appear to be expressed in these conditions. In contrast to these observations, the expression of MKK4 is not affected by either treatment (Fig. 3A). However, phosphorylation (activation) of MKK4 is specifically detected when the islets are incubated with cytokines, but not during isolation.

Next we investigated the implication of the upstream p38 kinases MKK3 and -6 in inducing p38 phosphorylation and activation during and after islet purification. Protein extracts from human islets were immunoblotted with an antibody recognizing both the phosphorylated forms of MKK3 and -6 together, as well as with an antibody recog-

nizing MKK3 alone (Fig. 3B). There are no marked changes in the ratio between the activated forms of MKK3/6 and expression of MKK3 during the first steps of the islets isolation procedure (whole pancreas). There was no increase in activity after purification and a 2.1-fold ($P < 0.05$ relative to cultured islets) increase after cytokine treatment. Collectively, whereas these results indicate that neither MKK3 nor -6 mediate activation of p38 during human islets isolation, two well-separated upstream pathways control JNK: cytokines specifically activate MKK4 in the absence of MKK7, whereas the isolation procedure recruits MKK7 only.

Recruitment of the NF- κ B-signaling pathway. NF- κ B is probably a major factor that controls β -cell death in autoimmune diabetes. We thus studied the consequences of islet isolation and cytokines on the activation of the NF- κ B pathway by analyzing NF- κ B DNA-binding activity in EMSA. NF- κ B was not detected immediately following the isolation procedure, was apparent at 12 h of culture, and reached maximum levels after 36 h. No NF- κ B DNA-binding activity was detected after 48 h of culture (Fig. 4A). These data correlated with lower levels of the inhibitor I κ B that increased at 48 h (data not shown).

Quantitative real-time PCR (LightCycler) was then used to measure iNOS mRNA expression throughout the islet isolation and after culture (Fig. 4B). iNOS expression was not detected during the isolation process. However, iNOS mRNAs were detected after 48 h of culture and persisted for at least 4 days (data not shown). iNOS induction could only marginally be further increased when islets were treated for 1 h with the three cytokines, indicating that iNOS expression following isolation reached levels comparable to those obtained after cytokine treatment.

High-level apoptosis following islet isolation. The very marked activation of the JNK- and p38-signaling pathways throughout the entire islet isolation procedure suggested that significant islet apoptosis should be detected at the end of the isolation process. Consistent with this, we indeed detected a majority of freshly purified islets staining positive in a TUNEL assay (Fig. 5). High apoptosis following isolation was already described (46). In contrast, none of the surviving islets after culture showed high-level DNA fragmentation as measured in the same assay. Note here that this isolation-induced apoptosis must be independent of iNOS expression (whose mRNA is not detected immediately after purification). In contrast, human islets treated with IL-1 β , in conditions that gave maximal activation of JNK (see above), did not show any significant sign of apoptosis (data not shown).

DISCUSSION

Islet transplantation has shown promise as a method to restore glucose homeostasis in type 1 diabetic patients (6,47). However, successful transplantation is limited by the availability of human islets and by a high loss of islets during isolation and following transplantation: up to 50% of the infused islet mass may be destroyed through apoptotic and other nonimmune inflammatory pathways, leading to graft failure (7). One of the critical factors in islet transplantation is early graft failure, which is defined as the immediate destruction of grafted islets (9,10). This tendency of islet grafts to fail to function within a short

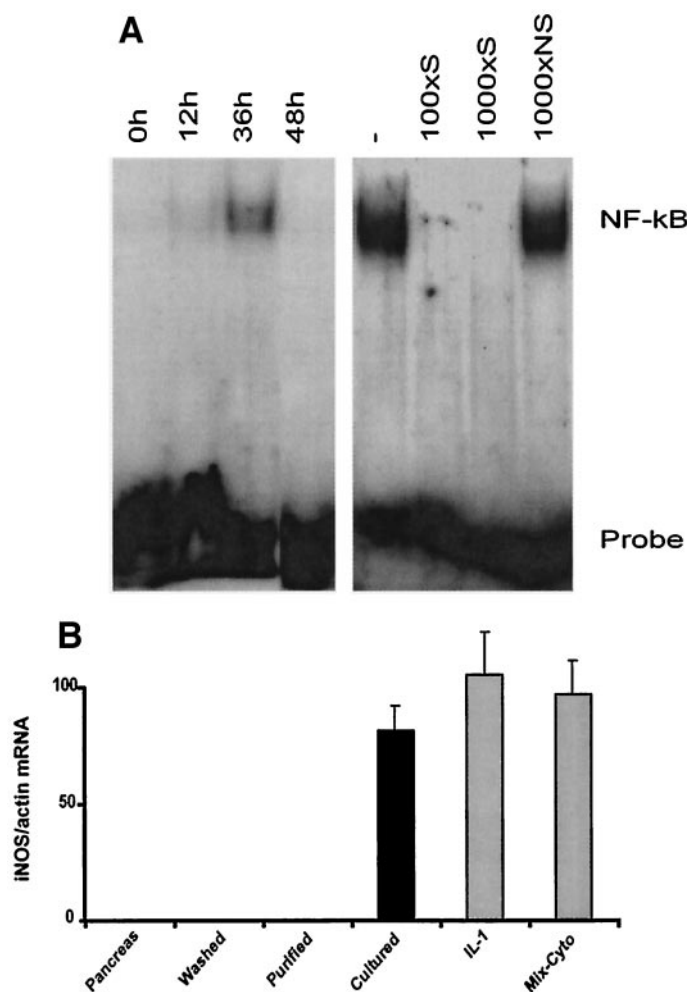


FIG. 4. Effect of the islet isolation procedure on NF- κ B DNA-binding levels and iNOS mRNA expression. **A:** NF- κ B levels were assessed by EMSA, as described in RESEARCH DESIGN AND METHODS. **Left panel:** Extracts were prepared from isolated islets at the indicated time points. **Right panel:** Competition studies with the indicated excess of specific (S) and nonspecific (NS) double-stranded oligonucleotides. Extracts for competition studies were from islets at 36 h of culture ($n = 3$). **B:** Human islets samples were taken at each main step of isolation (pancreas, digestion, wash, and purification). After culture (48 h), cells were exposed for 45 min to IL-1 β (IL-1; 10 ng/ml) alone or in combination with the cytokines TNF α and IFN γ (10 ng/ml). Total RNA was extracted and RT-PCR performed as described in RESEARCH DESIGN AND METHODS. The β -actin mRNA was used as an internal control. The illustration shown is representative of five experiments. \square , total pancreatic tissues (mainly exocrine, >98%); \blacksquare , purified endocrine tissues (>70%); \blacksquare , purified islets incubated with cytokines.

period after transplantation is termed PNF (48). These drawbacks indicated that transplantation of a sufficient number of islets, $\sim 6,000$ islet equivalents/kg body wt (1 islet equivalent = 150- μ m diameter islet), is an essential condition for a successful graft (49). The high level of β -cell loss subsequent to isolation makes this number of islets difficult to obtain from one single pancreas, making until recently the use of "double-donor" transplants to provide for an adequate therapeutic islet mass a prerequisite to successful grafting (50). Better pancreas preservation before islet transplantation remains therefore one of the potential improvements to overcome islet shortage and to improve PNF (46,51).

Isolation and purification expose islets to mechanical, enzymatic, osmotic, and ischemic stresses, the precise

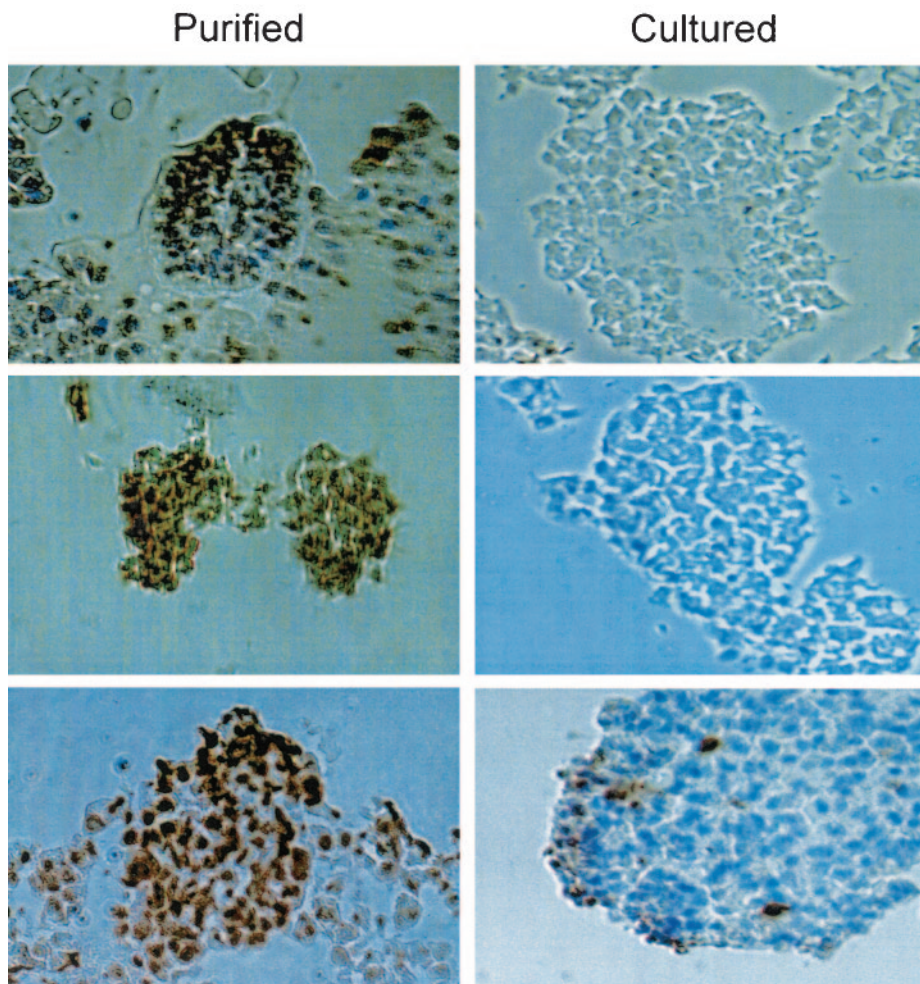


FIG. 5. TUNEL experiments. Purified islets were processed for TUNEL assay either immediately after isolation (*left*) or following 2 days of culture (*right*). Fragmented nuclei stain brown in the assay. *Left*: Examples of TUNEL-positive islets immediately following isolation. No such islets were detected after 48 h of culture.

consequences of which have been poorly characterized. We have shown here that the isolation procedure and cytokines recruit different stress-signaling pathways in human islets. Whereas isolation recruits the [MKK7 \rightarrow JNK/p38 \rightarrow *c-fos*] and the [NF- κ B \rightarrow iNOS] pathways, cytokines activate an [MKK4/MKK3/6 \rightarrow JNK/p38] pathway without recruitment of *c-fos*. Given the established importance of the [JNK/p38 \rightarrow *c-fos*] and the [NF- κ B \rightarrow iNOS] pathways in the apoptosis of insulin-secreting cells in experimental systems, these data indicate that islets sense the isolation procedure as an important stress recruiting the major proapoptotic intracellular pathways. It is also likely that the isolation procedure together with cytokines produced during the inflammatory process immediately following transplantation may further synergize to enhance cell death (Fig. 6) (52). This might be achieved at least partly through corecruitment of MKK7 by cytokines, as it is possible that MKK7 may also transduce cytokine signaling (as is the case in other cell types) (39).

The actual levels and activation states of the MAPK-signaling components inside islets before the isolation procedure are not clear from our data. This might be addressed by immunohistochemistry studies with the relevant antibodies, although data obtained in such a way are only semiquantitative. In any case, we cannot rule out that the JNK, p38, and NF- κ B pathways are already activated prior to isolation. This seems, however, particularly unlikely for the NF- κ B pathway, because we did not detect

any NF- κ B DNA-binding activity or iNOS expression at the end of the isolation procedure. Both DNA-binding and iNOS expression started after the completion of the procedure and were only transient. This strongly suggests that this pathway has been specifically recruited by the isolation procedure itself. Therefore, our data suggest that the isolation process may play a role in promoting β -cell death, although this will need to be experimentally demonstrated by using specific inhibitors of the JNK, p38, and NF- κ B pathways. In contrast, in cultured islets, all of these activities are markedly reduced, even though expression of iNOS is elevated. We would therefore suggest that it would be beneficial to culture islets before transplantation, probably in the presence of an iNOS inhibitor. The high number of TUNEL-positive cells detected immediately after isolation indicates that many islets die following processing. This suggests that only "nondamaged" islets will survive after culture, whereas the TUNEL-positive ones will ultimately disaggregate. This probably indicates a high heterogeneity of islet responses to stress events. Our hope would be that the manipulation of the aforementioned pathways during the course of isolation would decrease the number of TUNEL-positive islets at the end of purification, thus allowing for more viable islets to be transplanted.

In several cell systems, apoptosis necessitates a transcriptional activity at least partly mediated by the induction of the *c-fos* and *c-jun* genes (42,53). In this study,

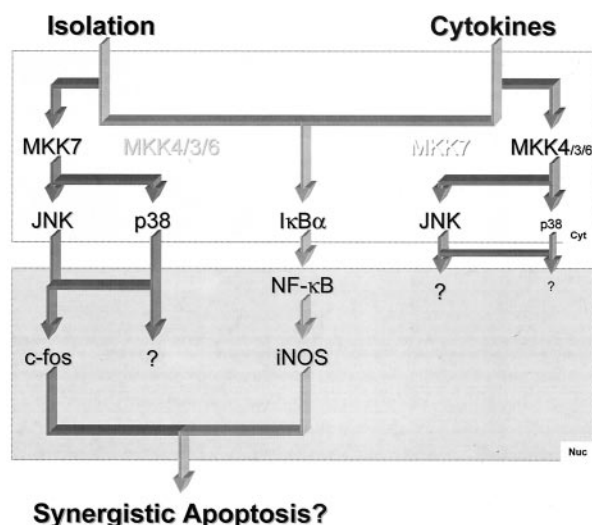


FIG. 6. Signaling pathways recruited during isolation and following cytokine attack. Schematic representation of the pathways implicated in islet loss during the isolation procedure and following cytokine attack. Isolation first recruits MKK7, but not MKK4 or -3/6, before activating JNK. It is not clear whether in some conditions (like simultaneous binding of MKK7 and p38 to the scaffold islet brain-2 (58,59) MKK7 might also activate p38. Following this, JNK and/or p38 signaling enter the nucleus and mobilize transcription of target genes such as *c-fos*. In such conditions, apoptosis may occur even in the absence of detectable iNOS. Following cytokine attack, MKK4 (but not MKK7, which is not expressed in resting islets) and to a lower extent MKK3/6 are recruited before activating JNK (strongly) and p38 (weakly). These events do not appear to be linked to classical nuclear events such as transcription of the *c-fos* gene. Our model postulates that in conditions leading to simultaneous *c-fos* and iNOS expression (i.e., when islets are transplanted immediately following isolation, a situation in which both the isolation and cytokine stresses are present), synergistic (high-rate) apoptosis would occur and might be a major contributor to PNF.

induction of *c-fos* has been observed only during isolation, but not when cultured islets were "acutely" (45 min) treated with IL-1 β or with a mix of cytokines. Both stresses recruited JNK to the same high level, but cytokines recruited p38 less markedly. Specific retention of JNK signaling in the cytoplasm has been already described (54); our data indicate that this particular behavior might be restricted to cytokine stresses. The absence of a marked expression of *c-fos* by cytokines therefore appears to be very specific for islets (including human islets). Assuming that apoptosis of human islets probably requires, although not demonstrated, at least some JNK- or p38-mediated nuclear events (i.e., *c-fos* expression), we would hypothesize that any JNK- and/or p38-mediated apoptosis is primarily elicited during the isolation procedure.

The upstream pathways leading to JNK activation also appear to diverge depending on whether the initiating stress is from isolation or cytokines: MKK7 probably mediates JNK activation during isolation, whereas MKK4 is specifically recruited by cytokines. Both MKK4 and -7 are abundantly expressed in exocrine tissues and in purified islets. Remarkably, MKK7 becomes undetectable in cultured islets. As MKK4 is not recruited during isolation, specific expression of MKK7 in islets during the procedure appears to be the crucial upstream mediator of JNK activation. The expression of MKK7 after purification may sensitize islets to cytokine attack (as described for other cell systems) (34) if islets are to be transplanted immediately after isolation. This might increase damage of func-

tional islet mass and contribute to early graft failure and PNF. Therefore, the observed downregulation of MKK7 expression might represent another extremely important beneficial consequence of culturing islets before transplantation.

The deleterious effect of NO on mediating β -cell toxicity prompted us to explore activation of NF- κ B, the major transcription factor responsible for de novo transcription of iNOS and concomitant elevated production of NO (55). The combination of the three cytokines (IL-1 β , TNF α , and IFN γ) results in iNOS expression and increased production of NO (56,57). Here, we found that the isolation procedure results in a delayed accumulation of NF- κ B DNA-binding activity, concomitant with an accumulation of iNOS mRNA that was detected after 2 days of culture. In our hands, iNOS expression seems equally potently induced by the isolation procedure as by cytokines. However, because iNOS appeared to be expressed only well after the completion of the isolation procedure, iNOS probably does not play any significant role in the loss of islets during, or immediately following, purification.

In summary, the major characterized stress pathways known to mediate insulin-secreting cell death in animal models appear to be sequentially activated from the earliest stages of the human islet isolation procedure until the actual grafting. Pharmacological inhibition of the JNK, p38, and NF- κ B pathways from the beginning of isolation and throughout the transplantation procedure might prove critical for the maintenance of islet cell mass and the lowering of PNF (20–22), although this will need to be experimentally demonstrated. This could probably be best achieved through the use of cell-permeable peptides such as the JNK inhibitor JNKI (20), which aims to block the relevant pathways, as these molecules are able to remain in the islets after grafting (S.A., B.J.H., C.B., unpublished results). Our data would also strongly support the notion that culturing islets before transplanting them might improve survival through downregulation of the activity of these pathways.

ACKNOWLEDGMENTS

This study was supported by grants from the Botnar Foundation, Juvenile Diabetes Research Foundation International (nos. 4-2002-457, 598002, and 4-1999-841), the National Center for Research Resources, the National Institutes of Health (U42 RR016598-01), and the Swiss National Science Foundation (FNS 3200-65139.01). R.R. was supported by the Gebert R f Stiftung Foundation.

We thank Fabienne Maurer for always helpful discussion and critical reading of the manuscript.

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