

Response of Human Islets to Isolation Stress and the Effect of Antioxidant Treatment

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The process of human islet isolation triggers a cascade of stressful events in the islets of Langerhans involving activation of apoptosis and necrosis and the production of proinflammatory molecules that negatively influence islet yield and function and may produce detrimental effects after islet transplantation. In this study, we showed that activation of nuclear factor- κ B (NF- κ B) and poly(ADP-ribose) polymerase (PARP), two of the major pathways responsible for cellular responses to stress, already occurs in pancreatic cells during the isolation procedure. NF- κ B-dependent reactions, such as production and release of interleukin-6 and -8 and macrophage chemoattractant protein 1, were observed days after the isolation procedure in isolated purified islets. Under culture conditions specially designed to mimic isolation stress, islet proinflammatory responses were even more pronounced and correlated with higher islet cell loss and impaired secretory function. Here we present novel evidence that early interventions aimed at reducing oxidative stress of pancreatic cells and islets through the use of the catalytic antioxidant probe AEOL10150 (manganese [III] 5,10,15,20-tetrakis [1,3,-diethyl-2imidazolyl] manganese-porphyrin pentachloride [TDE-2,5-IP]) effectively reduces NF- κ B binding to DNA, the release of cytokines and chemokines, and PARP activation in islet cells, resulting in higher survival and better insulin release. These findings support the concept that the isolation process predisposes islets to subsequent damage and functional impairment. Blocking oxidative stress can be beneficial in reducing islet vulnerability and can potentially have a significant impact on transplantation outcome. *Diabetes* 53: 2559–2568, 2004

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EMSA, electrophoretic-mobility shift assay; HBSS, Hank's balanced salt solution; HP, human pancreatic isolation; HTK, histidine tryptophan ketoglutarate; IL, interleukin; MCP-1, macrophage chemoattractant protein 1; MnTDE, manganese (III) 5,10,15,20-tetrakis (1,3,-diethyl-2imidazolyl) manganese-porphyrin pentachloride (TDE-2,5-IP); NF- κ B, nuclear factor- κ B; PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species; TBST, Tris-buffered saline with Tween; TNF- α , tumor necrosis factor- α .

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To become more successful, allotransplantation of human islets requires the availability of less toxic antirejection protocols and larger quantities of usable islets (1). In addition, the islet isolation process remains inefficient, often yielding an insufficient mass of functional islet cells to achieve adequate function, even in the absence of immune-mediated rejection.

Many factors contribute to islet loss and insufficient β -cell metabolic function after transplantation (2–5). New data have shown that islet function is already undermined by stressful events before transplantation (6,7). These findings implicate the effects of organ explantation, cold storage time, and the isolation procedures themselves as potential threats to islets. The nonphysiological biophysical and biochemical ambient conditions that are present during organ harvesting and isolation require abrupt metabolic adaptation by islets, which may result in functional impairment and eventually cell death. Despite efforts to optimize the conditions of pancreas preservation *ex vivo* (8–10) and the islet isolation process to improve islet yield, only a significantly limited part of the islet pancreatic content survives the process of isolation and subsequent culture.

Although the cascade of events during pancreatic cell isolation that may cause β -cell dysfunction and death is not fully characterized, recent lines of research in rodents (11,12) and humans (13) have indicated that oxidative stress plays a major role in triggering the death of islets and surrounding exocrine tissue. Other reports have demonstrated that oxidative stress is strongly connected to the adverse effects of chronic hyperglycemia on insulin biosynthesis by islet β -cells (14–16).

It has been widely reported that islet β -cells are highly susceptible to oxidative stress because of their reduced levels of endogenous antioxidants (17–19). Under extreme conditions of stress, the islet antioxidant defenses may become overwhelmed, leading to a state of redox imbalance and the production of reactive oxygen species (ROS). One potential ROS-dependent target molecule is the nuclear transcriptional factor nuclear factor- κ B (NF- κ B). It is now known that NF- κ B is a key transcription factor involved in regulating proinflammatory cytokines, chemokines, adhesion molecules, and inflammatory enzymes. Blockage of NF- κ B, by administration of an NF- κ B decoy or use of antisense oligonucleotide treatment, protects

β -cells from the effect of interleukin (IL)-1 β -induced NO production (20,21). Furthermore, it has recently been demonstrated that the native enzyme manganese superoxide dismutase delivered to mouse islets by gene therapy approaches proved beneficial in improving islet cell survival after transplantation (22).

Stress induced during pancreas harvesting and cold storage contributes to lowering the quality of the islets. The additional effect of isolation stress on the generation of islet damage has not been fully investigated. In this study, we focused on the response of islets to isolation stress. We hypothesized that a number of cellular mechanisms occur at this stage that can lead to adverse effects on the final product and negatively influence islet survival. Our aim was to characterize the events that are linked to isolation and possibly identify ways to reverse their consequences. We found that NF- κ B activation as well as the generation of apoptosis and necrosis, involving the poly-(ADP-ribose) polymerase (PARP) pathway (23), occur during pancreatic islet isolation and persist throughout the culture. One interesting observation was that even under standard culture conditions, isolated islets produced and released cytokines and chemokines such as IL-6, IL-8, and macrophage chemoattractant protein 1 (MCP-1), molecules involved in cellular response to stress. The response of islets to stress could be further amplified by applying an *in vitro* model of chemical stress that reproduces the events occurring during the isolation process, ultimately causing islet dysfunction.

Antioxidative treatment with the antioxidant probe AEOL10150 (MnTDE; manganese [III] 5,10,15,20-tetrakis [1,3-diethyl-2-imidazolyl] manganese-porphyrin pentachloride [TDE-2,5-IP]) effectively reduced the levels of NF- κ B in treated islets. This phenomenon correlated with reduced production of select cytokines and chemokines and decreased activation of apoptotic and necrotic pathways, allowing for enhanced protection of islets. Our data demonstrated that isolation-induced stress causes islet responses that result in reduced functional capacity. Damage can be prevented, to a large extent, by antioxidant treatment that targets early cellular activation.

RESEARCH DESIGN AND METHODS

Donor characteristics and islet isolation. Human pancreata that were not allocated for whole organ transplantation were obtained from organ procurement organizations (Center for Organ Recovery and Education, Pittsburgh, PA, and National Disease Research Interchange, Philadelphia, PA) and harvested using standard multiorgan recovery techniques. The organs were perfused *in situ* via the abdominal aorta with cold histidine tryptophan ketoglutarate (HTK) or University of Wisconsin solution. Either HTK or University of Wisconsin solution was used for cold preservation of the pancreata. Islets obtained from 26 adult donors were used. Characteristics of the donors as well as the islet preparations are summarized in Table 1.

Pancreatic islets were isolated using the semiautomated method described by Ricordi et al. (24) with minor modifications. Pancreas dissociation was performed using multiple lots of Liberase (Liberase-HI; Roche, Indianapolis, IN). Enzymes were reconstituted and dissolved in cold (4°C) Hank's balanced salt solution (HBSS). Before being digested in a modified continuous digestion-filtration device, pancreata were intraductally injected with enzyme solution in a recirculation system designed by Lakey et al. (25). Islets were purified with a COBE 2991 cell separator using discontinuous Euro-Ficoll gradients; the purity was assessed by dithione staining, as previously described (13).

Antioxidant treatment. AEOL10150 (MnTDE; Incara Pharmaceuticals, Research Triangle Park, NC) was resuspended in HBSS at a concentration of 2 mmol/l. MnTDE was added as a supplement to culture or isolation medium at a final concentration of 34 μ mol/l. Exposure of islet cells to MnTDE was

TABLE 1
Donor and islet characteristics

Age (years)	49 \pm 15 (18–67)
Cold ischemia time (h)	10 \pm 2 (4–14)
BMI (kg/m ²)	29 \pm 6 (20–40)
Weight pancreas (g)	80 \pm 21 (48–121)
Digestion time (min)	23 \pm 9 (11–43)
IEQ	328,400 \pm 196,000 (50,000–575,000)
Purity (% islets/whole preparation)	74 \pm 13 (37–90)
IEQ/g	3,888 \pm 2,115 (534–8,928)

Data are means \pm SD (range) (n = 26 pancreas donors). For islet equivalent number (IEQ) data, yield is the result of 24 islet isolations.

carried out for a minimum of 30 min. In each experiment, treatment and control conditions were compared within the same donor tissue.

Islet culture. The islet preparations were cultured in bacteriological Petri dishes at 37°C in an atmosphere of 5% CO₂ in humidified air in CMRL-1066 (Gibco-BRL) medium supplemented with 10% FCS, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mmol/l L-glutamine (Life Technologies, Grand Island, NY) (26). The islets were cultured for a maximum of 7 days, with the medium being changed every second day. For studies involving islet survival, insulin release, and cytokine-chemokine production, the islets were hand picked using a dissecting microscope. In summary, groups of 50 hand-picked islets (diameter 150–250 μ m) were randomly assigned to control and experimental groups. Each batch was subcultured in 35 \times 10-mm Falcon dishes at a concentration of 25 islets/ml for 60 h. In the treated groups of islets, MnTDE was added to the medium at a concentration of 34 μ mol/l. To determine mass, islets were counted by two independent investigators at the beginning and end of the observation period. Variability of <1% was determined between counts. Samples of culture supernatant were collected and stored at –80°C for basal insulin release and cytokine and chemokine measurements.

Conditioned medium and chemical challenge. To subject the islets to stress that mimics the chemical effects occurring during digestion, we exposed the islets to conditioned medium as previously described (27). Groups of 100 cell aggregates (islets and acinar tissue) were hand picked and incubated at 37°C with a HBSS solution containing Liberase (1.4 mg/ml) for 1 h (27) in the presence or absence of catalytic antioxidants. Cells were washed with fresh culture medium several times to eliminate the Liberase residues and subsequently cultured for 24 h. The culture supernatant collected 24 h after enzyme exposure was subsequently used as conditioned medium. As control medium for these experiments, 100 aggregates were incubated in HBSS without Liberase.

Hand-picked islets of similar size (150–250 μ m) were cultured in groups of 50 in CMRL-1066 medium, supplemented with conditioned or control culture medium (ratio 1:1 with fresh, untreated culture medium) with and without MnTDE. After 60 h of culture, the supernatant was collected from all groups, the islets were counted, and cell attachment and other morphological differences were evaluated. Insulin concentration in culture supernatant was measured using an enzyme-linked immunosorbent assay (ALPPO, Windham, NH); cytokines (IL-1 β and -6 and tumor necrosis factor- α [TNF- α]) and chemokines (IL-8 and MCP-1) were quantified by Beadlyte Human Multi-Chemokine System 1 and Beadlyte Human Multi-Cytokine Beadmaster kits.

NF- κ B electrophoretic-mobility shift assay of human islets. Human pancreatic cells collected during the isolation procedure as well as cultured and conditioned medium-treated islets (islet-enriched fractions containing \geq 80% islets/whole tissue) were subjected to NF- κ B electrophoretic-mobility shift assay (EMSA). For nuclear and cytoplasmic protein extraction, the protocol was adapted from Tse et al. (28). The DNA-binding oligonucleotide and its corresponding complementary strand were used in these studies: 5'-AGTTGAGGGGACTTCCAGGC-3' (NF- κ B consensus). For supershift analysis, after a 20-min incubation of the oligonucleotide and the nuclear protein extract, 1 μ g of antibody against p50 (C-19; Cell Signaling, Beverly, MA) was added and the incubation was continued for an additional 15 min before electrophoresis.

Immunofluorescence and confocal microscopy. Sections of pancreatic tissue and purified, hand-picked cultured islets were fixed in 2% paraformaldehyde in Histo-Gel (Richard-Allan Scientific, Kalamazoo, MI) and Affigel/Bluegel (BioRad, Hercules, CA) and frozen.

Sections were cut onto gelatin-subbed slides, fixed in 2% paraformaldehyde, rinsed in PBS and 5% BSA, and blocked in 5% nonimmune goat serum for 45 min at room temperature. The primary antibodies used were rabbit anti-human IL-6 (#ab6672; Novus Biologicals, Littleton, CO; dilution 1:2,000),

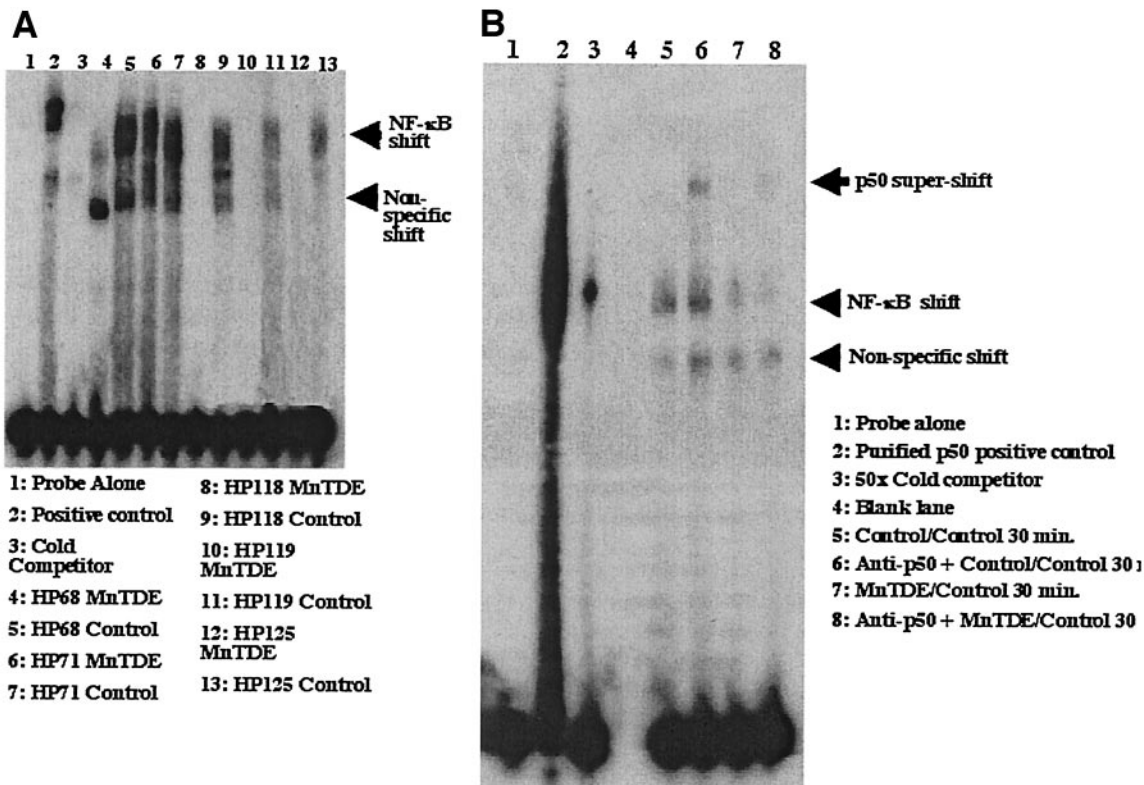


FIG. 1. EMSA. **A:** NF- κ B binding to a consensus κ B oligo is already evident in nuclear extracts of pancreatic cells during isolation (lanes 4–13). Human pancreatic tissue from the same donor was split into two parts during isolation; one-half was processed with isolation medium containing MnTDE, and the other half was exposed to MnTDE-free medium as control. Cold competitor lane consists of HeLa nuclear extracts incubated with 50-fold excess of unlabeled κ B consensus DNA binding oligo before 32 P-labeled κ B consensus oligo was added. Arrows denote the nonspecific and NF- κ B-specific shift. **B:** EMSA of NF- κ B in purified pancreatic islets after a 48-h culture. Groups of islets from the same donor were exposed to MnTDE-containing medium or maintained in standard culture medium for 30 min. The identity of the proteins in the slower migrating complex was identified by super-shift analysis with the addition of 1 μ g of antibody specific for p50. Arrows denote nonspecific shift, NF- κ B shift, and p50-shifted complex, as labeled on the gel.

rabbit anti-human IL-8 (#ab7747-500; Novus Biologicals; dilution 1:100), mouse anti-human MCP-1 (#550416, BD Biosciences Pharmingen, San Diego, CA; dilution 1:500), and mouse anti-human insulin (#I-2018; Sigma; dilution 1:3,000). Secondary antibodies used were goat anti-rabbit Cy3 (#111-165-144; Jackson ImmunoResearch, West Grove, PA; dilution 1:1,000) for IL-6 and IL-8, goat anti-mouse (#A-11026; Alexa 488; Molecular Probes, Eugene, OR; dilution 1:500) for insulin immunostaining, and goat anti-mouse Cy3-Fab (#115-167-003; Jackson ImmunoResearch; dilution 1:3,000) for MCP-1. Islets were viewed on an Olympus Fluoview BX61 confocal microscope (Melville, NY).

Western immunoblotting assays for poly(ADP-ribose) polymerase. Snap-frozen pancreatic cells or islet-enriched ($\geq 80\%$ purity) preparations were lysed in cold radioimmunoprecipitation assay buffer (50 mmol/l Tris-HCl [pH 8.0], 150 mmol/l NaCl, 1.0% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with protease and phosphatase inhibitors (1 mmol/l dithiothreitol, 1 mmol/l phenylmethylsulfonyl fluoride, 0.1 mmol/l sodium orthovanate, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin). Lysed islets were cleared by centrifugation (10,000g, 10 min, 4°C). Protein content of the extract was measured using a bicinchoninic acid assay kit (Pierce Biotechnology, Rockford, IL). Then 40 μ g of total protein content were electrophoresed on 15% SDS-PAGE, and the separated proteins were transferred onto an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was blocked with 5.0% nonfat dry milk in Tris-buffered saline with Tween (TBST; 0.15 mol/l NaCl, 10 mmol/l Tris-HCl, and 0.1% Tween 20 at pH 7.4) for 1 h at room temperature and then incubated with anti-PARP (Ab-2) antibodies (Oncogene, San Diego, CA; dilution 1:1,000) in blocking buffer for 2 h at room temperature. After being washed with TBST, the membrane was incubated with peroxidase-linked anti-goat IgG (Amersham-Pharmacia, Buckinghamshire, U.K. dilution 1:25,000) for 2 h. Detection was achieved using an enhanced chemiluminescence kit (Amersham-Pharmacia). Quantitation of Western blots by densitometry was performed with UN-SCAN-IT gel software (Silk Scientific, Orem, UT).

Statistical analysis. Statistical analysis was carried out using the Student's *t* test. *P* values < 0.05 were deemed statistically significant.

RESULTS

Islet response to isolation stress

NF- κ B nuclear translocation. NF- κ B DNA-binding was markedly reduced after islets and pancreatic cells were treated with catalytic antioxidants. To assess baseline levels of stress during isolation as well as during islet culture, we measured NF- κ B binding by EMSA using a consensus NF- κ B oligo. Samples analyzed included 1) pancreatic cells during isolation, in the presence or absence of MnTDE, and 2) isolated islets cultured in the presence or absence of MnTDE. In Fig. 1A, it is evident that during islet isolation, the transcription factor NF- κ B translocated to the nucleus of pancreatic cells. A marked reduction in NF- κ B binding, however, was found in cell samples exposed to MnTDE, as compared with untreated cells from the same donor pancreas. EMSA showed a clear organ-to-organ variability that may have reflected different levels of stress and consequently different conditions of the islets in relation to individual donors. For example, NF- κ B binding to the consensus oligonucleotide was greater in islets from human pancreatic isolation (HP)68 than in those from HP125 (Fig. 1A). Nonetheless, regardless of the intrinsic degree of activation (donor-to-donor variability), in all samples studied, NF- κ B activation was reduced when oxidative stress was quelled by MnTDE treatment.

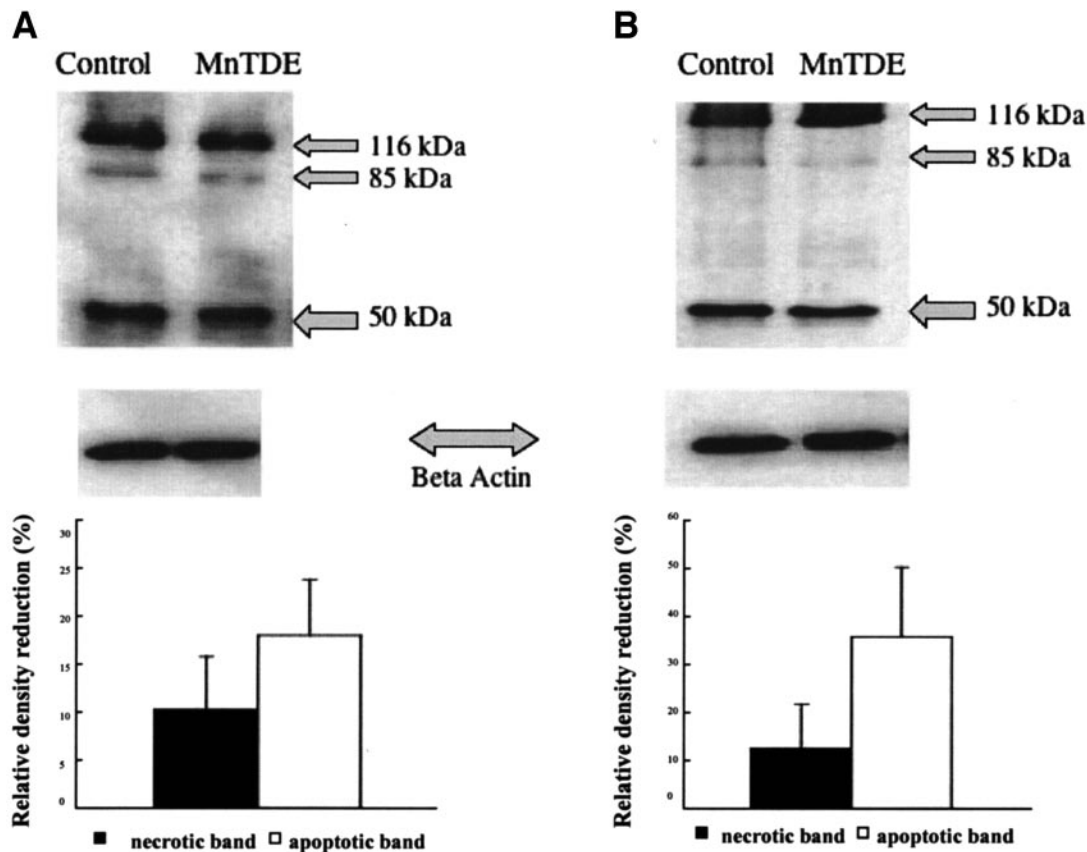


FIG. 2. PARP cleavage fragments (indicative of ongoing cell damage and death) were found in pancreatic cells during isolation and in purified islet preparations in culture. Western blot gels, representative of five independent experiments, show the fragments of 85 and 50 kDa (typical of apoptosis and necrosis, respectively) in addition to the 116-kDa full-length PARP band. The 1-h exposure of cells to catalytic antioxidants during isolation resulted in a reduction of both apoptotic and necrotic cleavage products (A). Purified islets maintained in culture for 60 h in MnTDE-enriched medium showed more accentuated reduction in apoptotic and necrotic by-products than untreated control groups (B). Band density reduction (percent) after MnTDE treatment is shown in the bar graphs as means \pm SE of five experiments. A statistically significant reduction in apoptotic band density was evident after MnTDE treatment ($P < 0.02$ for isolation, $P < 0.04$ for culture).

Even 2 days after isolation, NF- κ B activation was detected in purified isolated islets maintained under standard culture conditions. Exposure to MnTDE (Fig. 1B) was associated with lower NF- κ B binding activity. The samples were supershifted with an antibody recognizing the p50 subunit of NF- κ B. A clear shift was demonstrated from control medium-treated islets (Fig. 1B, lane 6). The complex was incubated with the anti-p50 antibody 15 min before separation on the nondenaturing acrylamide gel. Figure 1B (lane 5) represents the control medium-treated islets without the addition of the anti-p50 antibody, thus demonstrating the primary shift. Islets incubated in the presence of MnTDE demonstrated a reduction in the basal levels of NF- κ B (lane 7). This reduction in the primary binding was confirmed by the absence of a strong supershifted band (lane 8) when incubated with the anti-p50. Furthermore, we determined that the lowest band on the gel (Fig. 1B) represented a nonspecific shift, because when the samples were subjected to supershift with the anti-p50 antibody, the nonspecific band's intensity remained constant, unlike the NF- κ B-dependent band intensity, which diminished with the shifting of the p50 subunit. We recently described (28) that MnTDE inhibits the binding of the p50 subunit to DNA in bone marrow macrophages; therefore, we believe that this is the mechanism of reduced binding that we saw in the MnTDE-treated islets.

We have determined by supershift assay that p50 is one of the components of the complex. We speculate that the other component that makes up the NF- κ B complex is p65, forming the fully active p50-p65 heterodimer, as this heterodimer is the most commonly found complex and is capable of actively binding DNA, leading to the transactivation necessary for proinflammatory cytokine expression. Furthermore, the supershift revealed a remaining band in the primary shift; therefore, it is unlikely that the components that are present are p50-p50 homodimers. It is also known that p50-p50 homodimers cannot lead to transactivation, leading to recruitment of RNA polymerase for transcription.

Western blot analysis of PARP cleavage. PARP degradation by caspase activity is considered a biochemical marker for apoptosis as well as necrosis (29,30). The same polyclonal antibody recognizes the 116-kDa intact PARP fragment as well as the 85- to 90-kDa proteolytic fragments that characterize apoptosis and minor cleavage products of 35–50 kDa that are typical of necrosis. PARP cleavage was evaluated in pancreatic cell extracts during isolation (Fig. 2A) and in purified islets during culture (Fig. 2B), in the presence or absence of MnTDE treatment. The data showed that PARP degradation had already occurred during isolation, indicating that apoptosis and necrosis were ongoing. A comparison of the immunoblots of islets

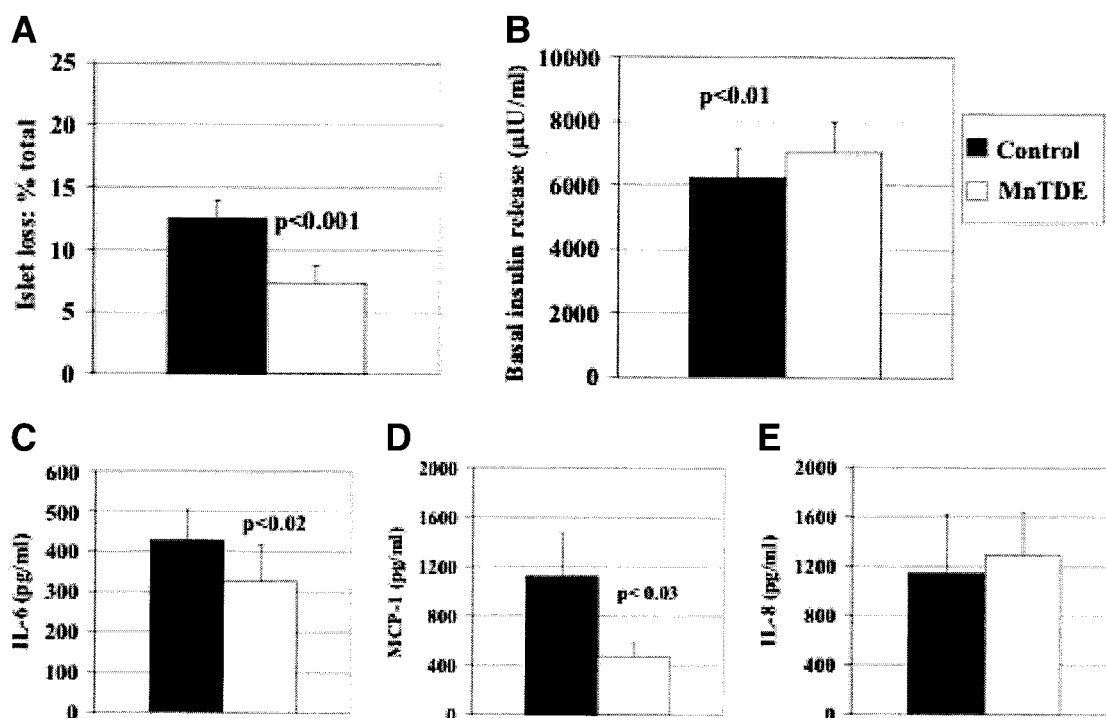


FIG. 3. *A*: Islet loss determined 60 h after standard culture conditions with (□) and without (■) MnTDE. Islet loss is indicated as percent of the total ($n = 12$). $P < 0.001$ for control versus MnTDE-treated groups (consisting of purified hand-picked islets). *B*: Basal insulin release by purified hand-picked islets after a 60-h culture in the presence or absence of MnTDE ($n = 16$). $P < 0.01$ for control versus MnTDE-treated groups. *C–E*: Concentrations of IL-6, MCP-1, and IL-8 detected in the culture medium of hand-picked islet groups after culture with and without MnTDE ($n = 13–18$). Data are means \pm SE.

and pancreatic digests showed that a more pronounced cleavage of PARP was found in untreated control islets (those that exhibit higher levels of 85- to 90-kDa as well as 35- to 50-kDa PARP by-products) when compared with MnTDE-treated islets. Quantification of the differences in band density is reported in the bar graphs at the bottom of Fig. 2.

Postisolation islet functional characteristics. Islet survival in culture was quantified by counting the number of islets at the beginning and end of the observation period. The initial phase of the process that eventually leads to islet destruction during culture is characterized by adherence of the islets to the surface of the dish. Usually degradation occurs quickly, involving the whole islet unit. Both free-floating and attached islets were counted. We noticed that untreated control islets tended to adhere to the bottom of the dish more often than antioxidant-treated islets (data not shown).

Figure 3A shows that ~12–14% of the islets were lost during the 60-h observation period. The addition of antioxidants reduced the loss by half, to ~7%. As shown in Fig. 3B, slightly higher, yet statistically significant, insulin concentrations were found in the culture medium of MnTDE-treated islets after the 60-h incubation, in comparison with control islets. The concentrations of insulin were normalized for the total islet number at each condition, which differed in relation to the treatment.

Microenvironmental cytokines and chemokines. Measurable concentrations of cytokine and chemokines were detected in the culture medium of hand-picked islets after 60 h (Fig. 3). IL-6 accumulated in the culture medium at a concentration of ~400 pg/ml in control

cultures, whereas lower values ($P < 0.02$) were measured in MnTDE-treated islet culture medium (Fig. 3C). MCP-1 was found in control and, in lower amounts ($P < 0.03$), in MnTDE-treated cultures (Fig. 3D). IL-8 was detected in all conditions, but its concentration was not influenced by the addition of catalytic antioxidants (MnTDE) (Fig. 3E). IL-1 β and TNF- α concentrations were also measured, but their levels were below the detection limit (data not shown).

Response of islets to artificially induced isolation stress. To assess whether isolation stress is directly involved in the proinflammatory response of isolated islets, we designed an in vitro experiment aimed at mimicking such stress. One of the major events that characterizes the isolation procedure is the exposure of islets to proteolytic enzymes and by-products generated by pancreatic cells undergoing isolation. Conditioned medium was produced by exposing islets and acinar cells to isolation enzymes with and without catalytic antioxidants; after Liberase was washed out, it was used as culture supplement to different groups of islets.

NF- κ B nuclear translocation. Figure 4 shows NF- κ B translocation in isolated islet-enriched fractions treated with conditioned medium made with and without catalytic antioxidants. NF- κ B binding to the consensus oligo was lower in islets incubated with conditioned medium made in the presence of MnTDE (Fig. 4, group 6) as compared with conditioned medium generated in the absence of MnTDE (Fig. 4, group 7). Two different islet preparations (from HP104 and HP107) are shown. Again, slightly different patterns were seen in the EMSAs; there were some nonspecific bands, depicted in Fig. 4 as nonspecific shift.

Islet functional characteristics. Islet loss was higher

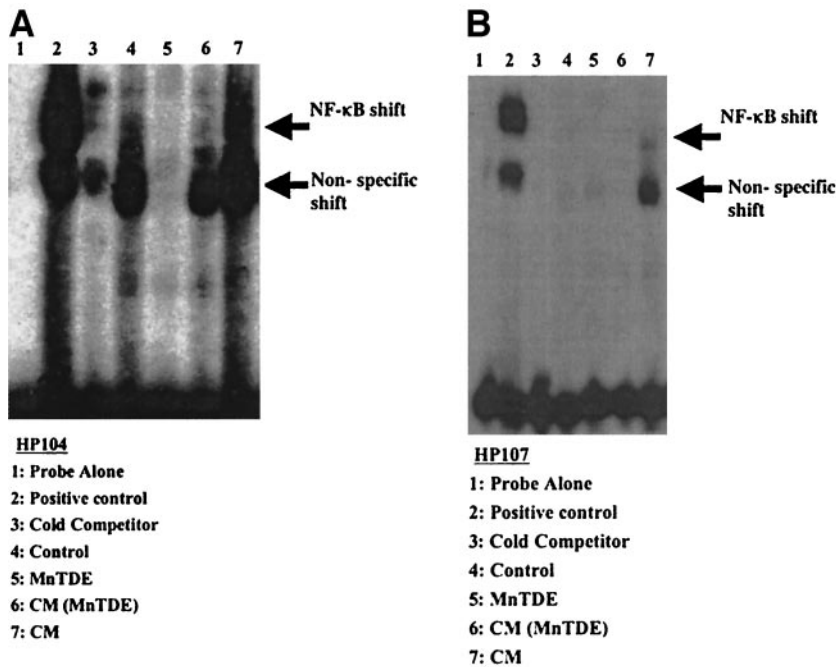


FIG. 4. EMSA of nuclear extracts of isolated islets cultured for 30 min with conditioned medium (CM) previously made in the presence or absence of MnTDE. Two different donor preparations were used: HP104 (A) and HP107 (B). Control, untreated islets; MnTDE, islets exposed to MnTDE; CM (MnTDE), islets exposed to conditioned medium made in the presence of MnTDE; CM, islets exposed to conditioned medium.

after culture with conditioned medium. However, the addition of catalytic antioxidants during the preparation of the conditioned medium reduced islet loss. This reduction in islet loss was more evident in those groups where antioxidative treatment was continued during the 60-h culture period (Fig. 5A).

The presence of conditioned medium was associated with a clear-cut decrease in basal insulin release in the culture medium, where insulin concentrations were 20% lower than those in control (Fig. 5B). It was interesting to see that the addition of conditioned medium produced in the presence of MnTDE did not efficiently reduce insulin concentration in the culture medium. Heat inactivation of conditioned medium prevented the reduction in insulin release (Fig. 5B), indicating that the biological activity of the conditioned medium was sensitive to temperature.

Microenvironmental cytokines and chemokines. After islets were incubated with conditioned medium, IL-6, MCP-1, and IL-8 concentrations increased (Fig. 5C–E). Because the cytokine and chemokine content of the conditioned medium itself contributed only marginally to the measured concentration (data not shown), the rise was attributed to ex novo production and release. Supplementation of antioxidants resulted in significantly lower IL-6 and MCP-1 concentrations. IL-8 levels, however, as noted previously, peaked irrespective of catalytic antioxidant treatment. IL-1 β and TNF- α concentrations were under the detection limit (data not shown).

Immunofluorescence analysis. The presence of cytokines and chemokines in the culture medium of highly purified islets raised a question as to which cell types were responsible for the synthesis of the proinflammatory molecules. Immunofluorescence techniques were used to stain pancreatic tissue collected before isolation and samples of hand-picked islets with IL-6, IL-8, and MCP-1 antibodies. Figure 6A, D, and G illustrate islets exposed to conditioned medium containing insulin-positive cells as well as cytokine- and chemokine-positive cells. As shown, many MCP-1- and IL-6-positive cells in isolated islets

were also positive for insulin (Fig. 6A), whereas IL-8 stained non- β -cells. Figure 6B, E, and H show islets exposed to conditioned medium made in the presence of MnTDE. Multiple sections from three different pancreatic organs were also analyzed before the isolation process was initiated. Figure 6C, F, and I show that insulin-positive cells before isolation did not react with any of the three cytokine- or chemokine-specific antibodies. In the intact pancreas, IL-6- and IL-8-positive cells were not islet β -cells.

DISCUSSION

The metabolic consequences of isolating human islets from the pancreas and the degree of their survival and function after clinical transplantation are not well known. Studies indicate that cold storage of the pancreas after explant from cadaveric donors and the process of chemical and mechanical digestion used for isolation can be harmful to the islets (6,7).

Other than the direct effects on islets, we have limited information on the modifications of acinar and other pancreatic nonislet cells during the process of isolation. Considering that the islet preparations used for clinical transplantation contain, in addition to islet β -cells, considerable amounts of contaminant pancreatic nonislet cells, attention should also be devoted to studying the evolution of each cellular component of the islet graft and how different cells interplay in the islet microenvironment after isolation and transplantation.

Recent studies have shown that the stress evoked by isolation increases the expression of cytokine and chemokine genes in human islets (31). It has also been reported in rodent studies that pancreatic acinar cells can be involved in inflammatory responses, because exocrine tissue is capable of producing and releasing cytokines in response to isolation stress (12).

In this study, we provided evidence that cytokines and chemokines are produced and secreted by isolated human

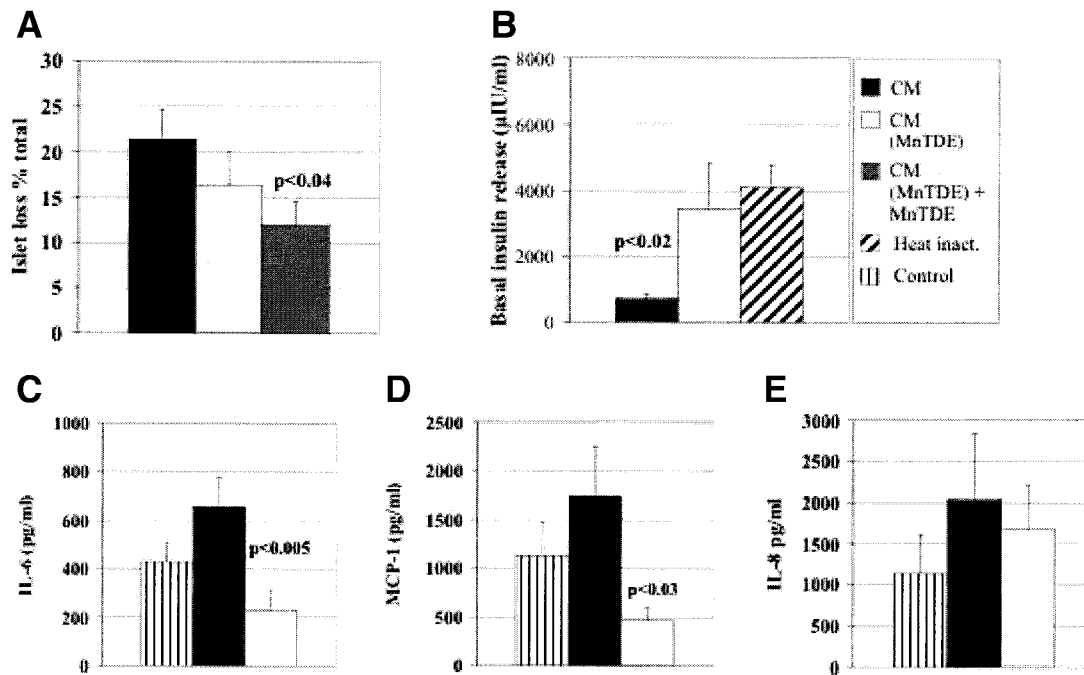


FIG. 5. *A:* Islet loss after exposure to conditioned medium (CM). Conditioned medium treatment caused >20% islet loss (■). When conditioned medium was made in the presence of MnTDE (□), islet loss was reduced. Further MnTDE supplementation of the culture medium (▨) made the difference in islet loss statistically significant ($n = 6$; $P < 0.04$ for CM vs. CM [MnTDE] + MnTDE). *B:* Basal insulin release by purified hand-picked islets after 60-h culture with conditioned medium. Significantly higher insulin concentration was measured when the islets were exposed to conditioned medium made in the presence of MnTDE ($n = 5$); $P < 0.02$. Heat inactivation efficiently prevented conditioned medium-mediated effects on insulin accumulation. CM (MnTDE), conditioned medium made in the presence of MnTDE; CM (MnTDE) + MnTDE, addition of MnTDE during the preparation of conditioned medium and as supplement in the islet culture medium. *C–E:* Exposure to conditioned medium increased cytokine and chemokine release in the culture medium of purified hand-picked islets. The graphs show accumulation of IL-6, MCP-1, and IL-8 after 60 h of culture in the presence or absence of MnTDE ($n = 12$). IL-6: $P < 0.005$ for CM vs. CM (MnTDE). MCP-1: $P < 0.03$ for CM vs. CM (MnTDE). IL-8: difference between CM and CM (MnTDE) groups was not significant. In *C–E*, control bars report chemokine-cytokine concentrations measured in islets untreated with CM (also shown as control in Fig. 3C–E).

islets during culture and, to a higher extent, after elicited stress. The release of proinflammatory molecules in the microenvironment of isolated islets raises justified concerns, considering the wide clinical application of islet transplants, as the same islet β -cells are known to be very sensitive to the detrimental effects of cytokines (32–36). Beyond a direct toxic effect on β -cells, it has also been reported that IL-6 interacts with a cascade of proinflammatory cytokines to induce hepatic production of serum amyloid-A, one of the major mediators of atherosclerosis (37). Chemokines such as MCP-1 and IL-8 may further jeopardize islet function after transplantation by enhancing recruitment and activation of host macrophages and leukocytes at the implant site. In the context of intraportal islet transplantations, these mechanisms may play a crucial role in the triggering of graft dysfunction and the eventual loss of islets. The clinical relevance of such events has been shown by a recent report demonstrating that human islet cells are able to attract monocytes and macrophages and that MCP-1 plays a relevant role in achieving long-term insulin independence in patients (38).

The role of isolation stress appears to be central in eliciting a proinflammatory response by the isolated islets. Although a new design for less aggressive methods finalized to islet isolation is not yet available, novel approaches specifically aimed at preventing damage that occurs during such procedures should be identified and examined. The efficacy of blocking oxidative stress (and proinflammatory

responses) as a way to enhance islet β -cell survival has been supported by our group's work (13,22) as well as by that of others (11,39–42). In particular, our group has shown that islets cultured in the presence of catalytic antioxidants exhibit higher survival after transplantation of marginal islet masses in mice (13). These encouraging results call for more experiments aimed at exploring possible clinical applications.

However, no clear information has been obtained on the mechanisms involved in the generation of the damage and, consequently, on possible ways to infer islet protection. Our data showed that NF- κ B nuclear translocation occurred in pancreatic cells during the isolation process and was maintained during culture. Moreover, catalytic antioxidants efficiently inhibited NF- κ B DNA binding. This became particularly evident when pancreata were split and processed in parallel with and without antioxidants. At the isolation stage, apoptotic and necrotic pathways mediated by PARP activation were also detected, and, analogous to what was observed for NF- κ B, were sensitive to protective antioxidant treatment. It is our assumption that blocking apoptotic and necrotic pathways at an early stage may have positive consequences for the long-term survival of the islets. The expression of NF- κ B can be considered a triggering factor for the production of proinflammatory cytokines and chemokines. Their induction, in turn, contributes to islet dysfunction by cyclical amplification of the oxidative stress cascade involving more

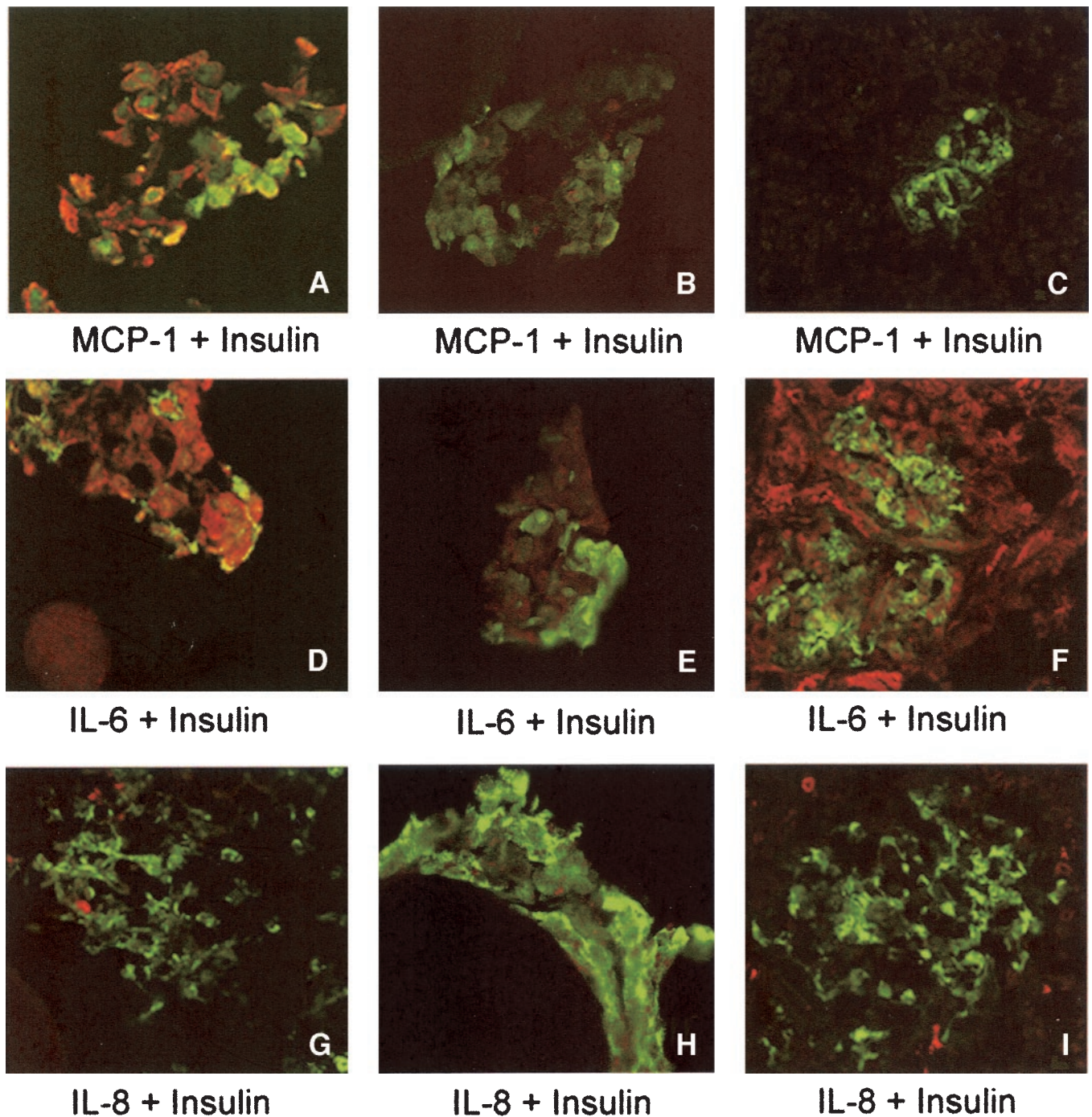


FIG. 6. Confocal images of hand-picked islets exposed to conditioned medium (*A*, *D*, and *G*) or conditioned medium made in the presence of MnTDE (*B*, *E*, and *H*). Pancreatic tissue before isolation is shown in *C*, *F*, and *I*. Green fluorescence indicates insulin-positive cells. Red fluorescence characterizes MCP-1 (*A–C*), IL-6 (*D–F*), and IL-8 (*G–I*)-positive cells. Magnification $\times 60$, zoom $\times 2$.

inflammatory mediators, resulting in a larger loss of β -cell functional mass (17,43). We found that the proinflammatory cytokine IL-6 and the chemokines IL-8 and MCP-1 accumulated in the islet culture medium after isolation and reached higher concentrations after chemical stress. Reduced NF- κ B translocation, consequent to antioxidative treatment, was correlated to reduced IL-6 and MCP-1 concentrations in the culture medium. IL-8 secretion, however, was not correlated to NF- κ B activation, neither was it reduced by antioxidative treatment, alternatively

suggesting that NF- κ B-independent mechanisms not affected by antioxidants regulate its production and secretion, as has also been suggested by others (12).

In this study we also addressed whether islets, and in particular β -cells, contribute to the production of cytokines and chemokines in the microenvironment. Immunofluorescence staining of highly purified islets with IL-6, IL-8, MCP-1, and insulin-specific antibodies showed that islet non- β -cells reacted with IL-8 antibodies, whereas a number of β -cells (insulin-positive cells) reacted with IL-6

and MCP-1 antibodies. These data confirmed and further expanded the finding that human islets can express genes for proinflammatory cytokines (31). Our results are therefore suggestive of a possible involvement of the β -cells themselves in the production of IL-6 and MCP-1, whereas islet non- β -cells appear to be involved in the production of IL-8. However, this conclusion still needs further confirmation. Early blockage of oxidative stress appears to be a key treatment; however, many of the mechanisms linked to subsequent islet response are already amplified during the isolation, suggesting that efforts should be made to prevent their activation up front to achieve higher islet protection.

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