Tissue Inhibitor of Metalloproteinase-1 Prevents Cytokine-Mediated Dysfunction and Cytotoxicity in Pancreatic Islets and β-cells

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In addition to inhibiting matrix metalloproteinase-2 and matrix metalloproteinase-9 activity, recent studies suggest that tissue inhibitor of metalloproteinase (TIMP)-1 may inhibit apoptosis in various cell lines. To address this question in pancreatic islets and β-cells, we treated rat pancreatic islets and INS-1 cells with a high-dose combination of the cytokines interleukin (IL)-1β, tumor necrosis factor-α, and interferon-γ with or without the addition of TIMP-1 and TIMP-2 protein. Using flow cytometry, we quantitated DNA fragmentation to assess cellular apoptosis and confirmed these observations with DNA laddering experiments. Next, we transfected the mouse TIMP-1 gene into INS-1 cells and performed Western immunoblotting to demonstrate expression of TIMP-1 protein. We treated TIMP-1–expressing INS-1 cells with high-dose cytokines and again used flow cytometry to assess DNA fragmentation. We also evaluated the effect of TIMP-1 on IL-1β–induced inhibition of glucose-stimulated insulin secretion (GSIS) in freshly isolated rat pancreatic islets. Finally, we evaluated the effect of TIMP-1 on inducible nitric oxide synthase (iNOS) gene expression and nuclear factor (NF)-κB activity in INS-1 cells stimulated with high-dose cytokines. TIMP-1 but not TIMP-2 prevented cytokine-induced apoptosis and cytokine-mediated inhibition of GSIS in rat islets and β-cells. TIMP-1 mediated these effects by inhibiting cytokine activation of NF-κB, but it did not affect nitric oxide production or iNOS gene expression. Therefore, TIMP-1 may be an ideal gene to prevent cytokine-mediated β-cell destruction and dysfunction in models of type 1 diabetes and islet transplantation rejection. *Diabetes* 50:1047–1055, 2001

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BSA, bovine serum albumin; EGFP, enhanced green fluorescent protein; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSIS, glucose-stimulated insulin secretion; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; JAK, Janus kinase; MMP, matrix metalloproteinase; NF, nuclear factor; PBS, phosphate-buffered saline; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor.

Matrix metalloproteinases (MMPs) are a family of secreted zinc proteases capable of degrading collagen and other matrix components. Approximately 21 members of the MMP family have been identified, and recent studies suggest that MMPs may participate in a wide variety of physiological and pathological responses (1). MMPs and tissue inhibitors of matrix metalloproteinases (TIMPs) are tightly regulated enzymes that coordinate matrix production and degradation. The obvious need for a dynamic process that regulates collagen turnover is evident in wound repair and remodeling (2,3). However, MMPs and TIMPs are involved in other significant but less obvious processes, such as tumor invasion and metastasis (4–7), inflammatory responses (8), embryonic development (9,10), arthritic diseases (11,12), and multiple sclerosis (13,14).

Recently, Herren et al. (15) discovered that certain metalloproteinases play a role in apoptosis. In particular, MMPs play a role in caspase activation by processing membrane-bound β-catenin to its active form via proteolysis. MMPs may also be involved in processing the transmembrane Fas ligand to the soluble Fas ligand (16). In addition, MMPs convert the membrane-bound form of pro–tumor necrosis factor (TNF)-α to the mature active inflammatory cytokine through proteolytic cleavage of a critical alanine-valine bond at amino acids 76 and 77 (17,18).

Alternatively, TIMP-1 overexpression prevented programmed cell death in a Burkitt's lymphoma cell line through both CD-95 (Fas)-dependent and -independent pathways. TIMP-1 overexpression upregulated Bcl-xL and decreased nuclear factor (NF)-κB activity, and the protective effect was not related to metalloproteinase inhibition (19). Furthermore, TIMP-1 expression has been used to prevent apoptotic cell death induced by hydrogen peroxide, adriamycin, and X-ray irradiation in human breast epithelial cell lines (20).

Pancreatic β-cells and islets are particularly sensitive to cytokine-mediated damage. Cytokine-treated rodent (and human) pancreatic islets demonstrate increased expression of the inducible nitric oxide synthase (iNOS) gene that in turn leads to nitric oxide (NO) production (21). NO inhibits glucose-stimulated insulin secretion (GSIS) and induces β-cell damage. Whereas inhibitors of iNOS, such as N-monomethyl-L arginine and L-N-arginine-methylester, can partially prevent cytokine-mediated β-cell damage through inhibition of NO production; NO-independent

pathways also exist. Several reviews have outlined in detail the prominent studies that relate cytokine-mediated β-cell damage with iNOS gene expression and NO production (22–25).

Some controversy exists as to the exact mechanism of pancreatic β-cell death in human type 1 diabetes and rodent models of this disease; however, most studies agree that apoptosis plays an important role in this pathological process (26–28). Overcoming cytokine-induced β-cell damage is a primary goal for protecting pancreatic β-cells from immune-mediated destruction in type 1 diabetes and islet transplantation rejection.

In the present study, we used the glucose-sensitive insulin-secreting rat β-cell line INS-1 for those experiments that would have required an extraordinary number of isolated rat islets or when transfection techniques could not be readily applied to isolated islets. When possible, we confirmed our finding in INS-1 cells with experiments using isolated rat pancreatic islets. We demonstrated that TIMP-1 overexpression in INS-1 cells or the addition of TIMP-1 protein to cultured rat islets dramatically inhibits high-dose cytokine-induced apoptosis by inhibiting NF-κB activity. In addition, TIMP-1 prevents cytokine-mediated inhibition of GSIS in rat pancreatic islets. TIMP-1–induced cytoprotection does not affect cytokine-induced iNOS gene transcription or NO production.

RESEARCH DESIGN AND METHODS

Reagents used in these experiments were as follows. RPMI-1640 medium was purchased from Life Technologies, Grand Island, NY. BCA reagent assay kit was purchased from Pierce Chemical, Rockford, IL. Recombinant human TIMP-1 and TIMP-2 were purchased from Chemicon International, Temecula, CA. Rabbit anti-human TIMP-1 polyclonal antibody was also purchased from Chemicon International. Horseradish-peroxidase–conjugated anti-rabbit IgG was obtained from Amersham Pharmacia Biotech, Piscataway, NJ. Mouse TIMP-1 plasmid (pBSmouse TIMP-1) was a generous gift from Dr. R.M. Schaefer, University of Muenster, Muenster, Germany. The Lipofectamine Plus transfection kit was purchased from Life Technologies. Collagenase Type XI used for islet isolation was obtained from Sigma Chemical, St. Louis, MO. Interleukin (IL)-1β (1 μg protein = 2×10^5 units), TNF-α (1 μg protein = 1.1×10^5 units), and interferon (IFN)-γ (1 μg protein = 2×10^4 units) were purchased from R & D Systems, Minneapolis, MN. Insulin assay kits were purchased from Linco, St. Louis, MO. NO was measured with a commercial kit obtained from R & D Systems. An Apoptotic DNA Ladder Kit was purchased from Roche Molecular Biochemicals, Indianapolis, IN. A Riboprobe in vitro transcription systems kit was purchased from Promega, Madison, WI, and was used for amplification of the iNOS mRNA probe.

Cell culturing. INS-1 cells were cultured to near confluence in RPMI-1640 medium with 11 mmol/l D-glucose supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mmol/l HEPES, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, and 50 μmol/l β-mercaptoethanol. All tissue culturing was performed in a Forma Scientific tissue-culture incubator that provided an environment of 95% O_2/5% CO_2 gas. Before addition of cytokines, the cells were depleted in RPMI-1640 medium with 11 mmol/l D-glucose plus 0.1% bovine serum albumin (BSA) for 18–24 h. The cells were gently washed in phosphate-buffered saline (PBS), and depletion medium was added back. At that time, TIMP-1 or TIMP-2 (25–200 ng/ml) was added in certain experiments 60 min before the addition of cytokines. IL-1β (5 ng/ml), TNF-α (30 ng/ml), and IFN-γ (30 ng/ml) were then added, and the cells were incubated for an additional 18 h. The cells were harvested for flow cytometry and DNA laddering experiments by using 1 ml 0.05% trypsin-EDTA solution. In addition, INS-1 cells transiently transfected with the plasmid pcDNA3.1/mouse TIMP-1 or plasmid pcDNA3.1 (see below) were cultured in a manner similar to that of nontransfected cells, and they received cytokine treatment for 18 h before harvesting for flow cytometry. All flow cytometry experiments were performed in triplicate or quadruplicate and repeated three to four times for reproducibility.

Islet purification and culturing. All animal studies were performed in accordance with guidelines set forth by the Research Animal Care Committee of City of Hope National Medical Center. Islet isolation and culturing techniques have been previously detailed (29). Male Sprague-Dawley rats weighing 250–300 g were used for islet isolation. Briefly, 30 ml cold Hank's buffer/Type XI collagenase solution was infused into the rat pancreatic duct via catheter. The inflated pancreas was removed, minced, and digested in a shaker-type water bath at 37°C. Islets were picked by hand under a microscope. Freshly isolated islets were aliquoted into sterile six-well plates (Sarstedt, Newton, NC) and cultured in RPMI-1640 medium containing 5 mmol/l glucose supplemented with 10% FCS, 10 mmol/l HEPES, and penicillin/streptomycin. The islets were allowed to equilibrate for 3 h, at which time GSIS studies were performed. For DNA laddering experiments, islets were cultured overnight in RPMI-1640 medium with 11 mmol/l glucose. The next morning, islet aliquots were transferred into six-well plates (200 islets per well) and treated with cytokines plus or minus TIMP-1 at the appropriate concentrations for 24 h.

Construction of mouse TIMP-1 plasmid and transient transfection into INS-1 cells. The full-length cDNA sequence that encodes mouse TIMP-1 was cut from plasmid pBSmouse TIMP-1 with the restriction enzymes *Bam*HI and *Hin*dIII. The resulting 825-bp insert was subcloned into the plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA).

One day before transfection, INS-1 cells were dispersed with trypsin-EDTA solution and counted. The cells were pipetted into 12-well dishes at a density of 1×10^4 cells per well so they would attain 70% confluence the next day. The pcDNA3.1/TIMP-1 plasmid (0.3 μg/well) was incubated with the Plus Reagent (5 μl/well) for 15 min at room temperature. Lipofectamine Reagent was diluted into medium without serum in a second tube according to the manufacturer's instructions. The precomplexed DNA and diluted Lipofectamine Reagent were mixed together and incubated for 15 min at room temperature. While lipid/DNA complexes were forming, the cell culture medium was removed and replaced with 500 μl transfection medium. Next, the DNA plus Lipofectamine Reagent mixture was added to each well (100 μl), and the cells were incubated at 37°C. After 3 h, the medium volume was increased to 2 ml with RMPI-1640 plus 10% FCS. The INS-1 cells were cultured for 18 h overnight, at which time the medium was changed to RPMI-1640 with 11 mmol/l D-glucose plus 0.1% BSA and incubated for an additional 18–24 h. Cytokines were added on the following day, and the cells were harvested after 18–24 h.

To demonstrate that INS-1 cells transiently expressed mouse TIMP-1, we performed Western immunoblots of cell lysates as described below. In addition, we performed similar transfection experiments with a pcDNA3.1/enhanced green fluorescent protein (EGFP) plasmid and counted EGFP-expressing cells versus total cell number in order to obtain an estimate of transfection efficiency (data not shown). We estimated our transfection efficiency at ~75%.

Western immunoblotting. INS-1 cells (~10^6) were lysed in a 1% Triton-0.1% SDS buffer in normal saline (pH 7.4) with 10 mmol/l HEPES plus 1 mmol/l EDTA and standard protease inhibitors. Cell lysates were centrifuged at 3,000*g* for 10 min, and a modified BCA protein assay was performed. Protein aliquots (100 μg per sample) were treated with Laemmeli sample buffer and then heated to >90°C for 5 min. Proteins were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P) in Tris-glycine buffer (pH 8.5) plus 20% methanol. The immunoblots were blocked overnight in 5% nonfat dried milk in Tris-buffer containing 0.1% Tween-20 and then washed with Tris-buffer. The blots were incubated for 2 h at room temperature with rabbit anti-human TIMP-1 polyclonal antibodies diluted 1:3,000 in Tris-buffer. The blots were washed and then incubated with horseradish-peroxidase–conjugated secondary antibodies at 1:2,000 dilution. The protein bands were visualized with enhanced chemiluminescence reagents (ECL Plus Western Blotting Detection System; Amersham Pharmacia Biotech).

DNA laddering assay. INS-1 cells and rat islets were treated with cytokines plus or minus TIMP-1, as described above. Cellular DNA isolation and DNA ladder detection was performed according to the manufacturer's instructions (Roche Molecular Biochemicals). DNA laddering was run on 1% agarose gels along with high- and low-molecular-weight markers.

NO determination. Nitrite measurement was performed as an indirect measure of NO production in rat pancreatic islets. The commercial kit we used (R & D Systems) included a nitrate reductase step that converts nitrate to nitrite. Freshly isolated islets (25 per well) were placed in 12-well dishes and IL-1β (0.1 ng/ml) plus or minus TIMP-1 (50 ng/ml) was added. The islets were incubated at 37°C for 48 h, at which time the medium was sampled and frozen for later NO determination.

Northern analysis. Rat iNOS RNA antisense template was purchased from Torrey Pines Biolabs, Torrey Pines, CA. We used an in vitro transcription system (Promega) to generate and amplify our P^{32}-labeled antisense RNA probe. The probe amplification was performed with T7-RNA polymerase according to the manufacturer's instructions. Total RNA was extracted with

TRIzol reagent (Gibco BRL, Grand Island, NY) from INS-1 cells cultured as previously described. We resolved 30 μg total RNA on a 1.2% formaldehyde/agarose gel. The samples were transferred to nylon membranes and hybridized with the ^{32}P-labeled RNA probe using Quikhyb Hybridization solution (Stratagene, La Jolla, CA) in a hybridization oven (Hybridizer HB-D; Techne). After hybridization and washing, nylon membranes were exposed to film (Biomax; Kodak) to create autoradiograms.

Because we were interested in the effects of TIMP-1 on IL-1β–induced inhibition of GSIS and high-dose cytokine-induced cytotoxicity (IL-1β + TNF-α + IFN-γ), we performed iNOS Northern blots on INS-1 cells subjected to both treatments. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for all experiments. The GAPDH RNA antisense template was obtained from Torrey Pines Biolabs. Quantitative densitometry was performed on autoradiograms with a GS 710 calibrated imaging densitometer and Quantity One 4.2.1 software (BioRad Laboratories, Hercules, CA). After obtaining density measurements, we calculated the iNOS-to-GAPDH ratio and normalized the lowest density value to 1 in each individual experiment.

Luciferase assay. An IL6–NF-κB luciferase reporter construct was a kind gift from Dr. J-J. Li (30). A plasmid containing the β-galactosidase gene driven by the cytomegalovirus promoter was obtained from Clontech Laboratories, Palo Alto, CA. INS-1 cells were co-transfected with the two plasmids using the lipofectamine (Life Scientific) method previously described. Transfected cells were preincubated with mouse TIMP-1 (50 ng/ml) for 1 h before treatment with high-dose cytokines IL-1β (5 ng/ml), TNF-α (30 ng/ml), and IFN-γ (30 ng/ml) for different time periods. Luciferase activity was measured with a luminometer (TD-20/20; Turner Designs, Sunnyvale, CA) using 100 μl whole-cell lysate and the same volume of luciferase assay reagent (Promega). An aliquot of the same cell lysate for each sample was used to measure β-galactosidase activity to normalize the luciferase activity. Luciferase assays were performed in triplicate and repeated twice for reproducibility.

Glucose-stimulated insulin secretion. Immediately after islet isolation, 20 rat islets per experiment were cultured for 3 h in 12-well dishes with 1.5 ml of RPMI-1640 medium with 5 mmol/l glucose plus 10% FCS, 10 mmol/l HEPES, penicillin *G* 100 U/ml, and streptomycin 100 μg/ml. Rat islets were pretreated with TIMP-1 (50 ng/ml) for 1 h before addition of IL-1β (0.1 ng/ml) and maintained overnight at 37°C. The next morning, the islets were gently transferred to RPMI-1640 medium with 3 mmol/l glucose and incubated for 1 h, at which time the medium was sampled for insulin measurement. The medium glucose concentration was then increased to 17 mmol/l and the islets incubated for an additional hour. Supernatants were frozen at −70°C and insulin assays performed at a later date. Each islet experiment was performed in triplicate and repeated three times for reproducibility.

Flow cytometry. The Analytical Cytometry Core at City of Hope National Medical Center uses a three-laser MoFlo Optical Bench cytometer. A Cytomation MoFlo analysis-sorter system was purchased for complex data analysis. Apoptosis assays were performed as follows. Trypsinized INS-1 cells were centrifuged and washed twice with PBS. The cells were counted, and 5×10^6 cells per experiment were incubated for 1 h at 37°C with Hoechst 33342 fluorescent dye (10 μg/ml). The cells were then processed (50,000 per experiment) by two-channel flow cytometry.

Statistical analysis. Statistical analysis of multiple comparisons was performed using analysis of variance with GraphPad Prism software (GraphPad Software, San Diego, CA).

RESULTS

TIMP-1 prevents cytokine-mediated apoptosis. The rat pancreatic β-cell line INS-1 was cultured to near confluence. Using a high-dose combination of cytokines IL-1β (5 ng/ml), TNF-α (30 ng/ml), and IFN-γ (30 ng/ml), we treated INS-1 cells plus or minus TIMP-1 or TIMP-2 for 18 h, at which time the cells were harvested and analyzed by flow cytometry. Using Hoechst 33342 fluorescent dye, we detected the percentage of cells with DNA fragmentation, as it is indicative of apoptosis. Figure 1 shows one representative flow cytometry experiment that was repeated three different times. In Fig. 1*A*, untreated INS-1 cells demonstrated 4.0 ± 1.0% low-molecular-weight DNA. Comparatively, cytokine-treated INS-1 cells demonstrated 33.2% ± 0.8% low-molecular-weight DNA (Fig. 1*B*). A single treatment with TIMP-1 (50 ng/ml) decreased the cytokine-induced DNA damage to 12.1 ± 1.8% ($P < 0.0005$ vs. cytokine-treated) (Fig. 1*C*), whereas a fourfold higher dose of TIMP-2 (200 ng/ml) significantly decreased DNA damage (24.3 ± 3.4%, $P < 0.05$) (Fig. 1*D*) but to a much lesser extent than TIMP-1. In addition, TIMP-1 (50 ng/ml) treatment alone did not affect the percentage of low-molecular-weight DNA (7.5 ± 0.4%) (data not shown).

To confirm that flow cytometry was accurately detecting DNA fragmentation, we performed DNA laddering experiments with INS-1 cells treated as previously described. In Fig. 2, *lanes 1* and *2* demonstrate classical DNA laddering when INS-1 cells are treated with the cytokine mixture. Cytokines plus TIMP-1 (50 ng/ml)–treated cells show complete inhibition of DNA laddering (*lanes 3* and *4*). *Lanes 5* and *6* show untreated control INS-1 cells, whereas *lanes 7* and *8* show EGTA-treated INS-1 cells (positive control). In addition, ~200 rat pancreatic islets per experiment were treated with cytokines plus or minus TIMP-1, and DNA laddering was performed. As seen in Fig. 3, TIMP-1 protected rat islets from cytokine-induced apoptosis (compare *lanes 3* and *4*).

We then took INS-1 cells and performed transient transfection experiments with the mouse TIMP-1 gene inserted in the vector pcDNA3.1. Again, we used Hoechst 33342 dye and our apoptosis assay to demonstrate that TIMP-1 overexpression totally prevented cytokine-induced apoptosis. As shown in Fig. 4, cytokines induced 20.9 ± 1.0% apoptosis in untreated INS-1 cells (*lane 1*). However, INS-1 cells containing pcDNA3.1/mouse TIMP-1 showed 2.9 ± 0.1% apoptosis when exposed to the same cytokine mixture (*lane 2*). Cytokine-treated INS-1 cells transfected with the pcDNA3.1 vector alone showed a small decrease in apoptosis (13.7 ± 2.3% [*lane 3*]), whereas the negative control INS-1 cells showed 1.9 ± 0.3% apoptosis (*lane 4*). In addition, INS-1 cells treated with pcDNA3.1 (*lane 5*) or pcDNA3.1/TIMP-1 (*lane 6*) plasmids demonstrated a small increase in apoptosis (5.7 ± 0.7 and 5.4 ± 0.4%, respectively) compared with untreated INS-1 cells, although it was not statistically significant. Therefore, TIMP-1 totally protected INS-1 cells from cytokine-induced apoptosis.

In Fig. 5, Western immunoblotting of protein lysates from pcDNA3.1/mouse TIMP-1–transfected INS-1 cells was performed. The figure shown is representative of two independent experiments. Using a polyclonal anti-human TIMP-1 antibody, we demonstrated a two- to threefold increase in TIMP-1 protein in transfected INS-1 cells (*lanes 2* and *3*) compared with control INS-1 cells (*lane 1*).

TIMP-1 prevents cytokine-induced rat islet dysfunction. We evaluated GSIS in rat islets treated with IL-1β (0.1 ng/ml) plus or minus TIMP-1 (50 ng/ml). As shown in Fig. 6, control untreated islets secreted 155 ± 5 ng insulin · h^{-1} · 20 $islets^{-1}$, whereas IL-1β–treated islets secreted 30 ± 4 ng insulin · h^{-1} · 20 $islets^{-1}$ ($P < 0.006$). Islets treated with IL-1β plus TIMP-1 secreted 118 ± 14 ng insulin · h^{-1} · 20 $islets^{-1}$ ($P < 0.06$ vs. control islets and $P < 0.02$ vs. IL-1β–treated islets), whereas islets treated with TIMP-1 alone did not show any alteration in GSIS compared with control islets (161 ± 14 ng insulin · h^{-1} · 20 $islets^{-1}$). Therefore, TIMP-1 was able to restore GSIS in cytokine-treated rat islets to 75% of their control value.

NO production in rat islets. As shown in Fig. 7, control rat islets generated 1.09 ± 0.06 μmoles of nitrite per 25 islets, whereas islets treated with IL-1β generated 1.98 ±

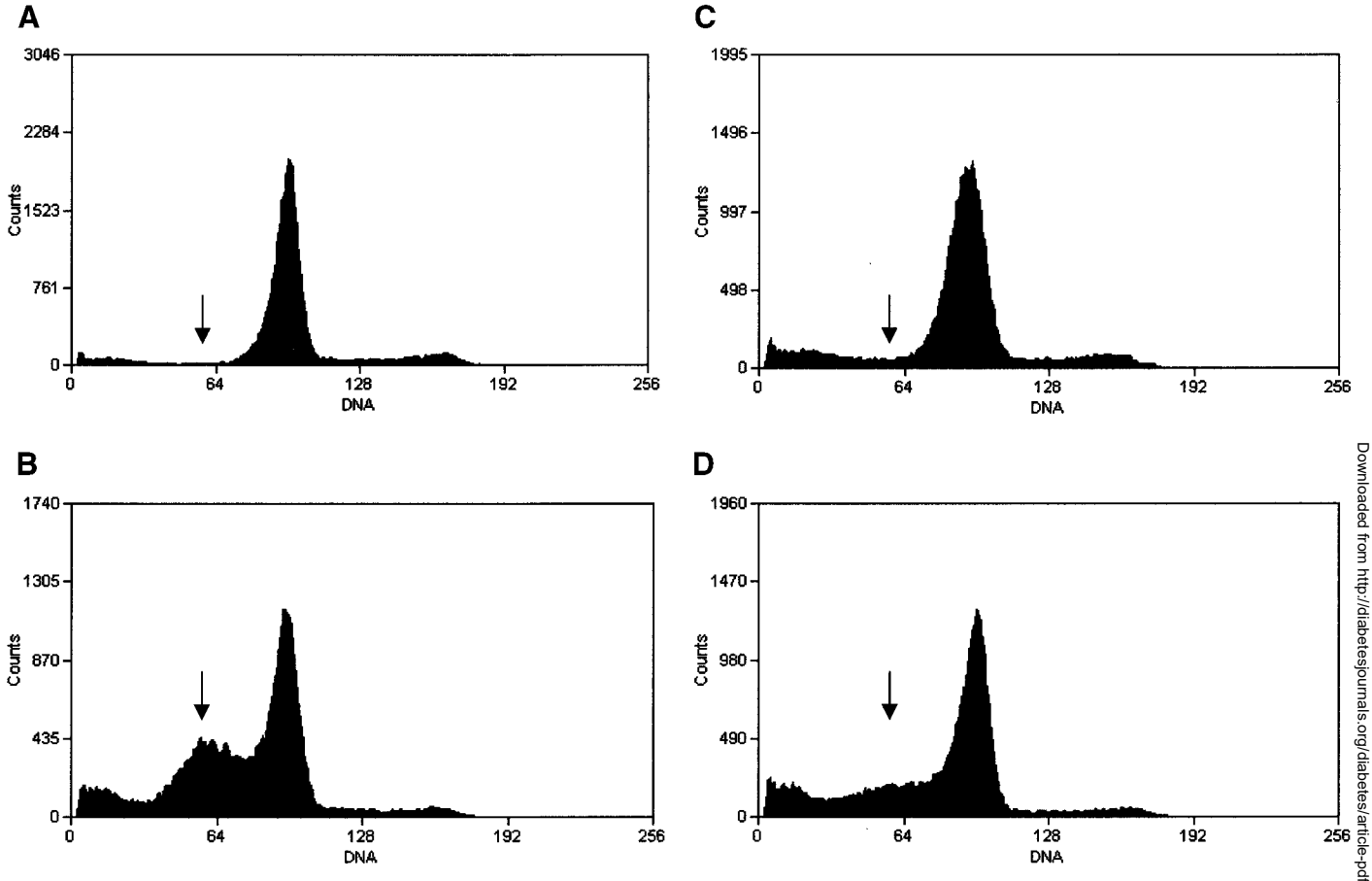

FIG. 1. Apoptosis assay using flow cytometry. Arrows indicate region of low-molecular-weight DNA. Untreated INS-1 cells demonstrated little DNA fragmentation (*A*), whereas cytokine treatment induced a high level of DNA fragmentation (*B*). TIMP-1 (50 ng/ml) treatment led to near total inhibition of cytokine-induced DNA fragmentation (*C*). A fourfold higher dose of TIMP-2 (200 ng/ml) decreased cytokine-induced DNA fragmentation (*D*) but was significantly less potent than TIMP-1. Each experiment was performed in triplicate and repeated three times for reproducibility.

0.1 μmoles of nitrite in 48 h ($P = 0.0006$ vs. control). Islets treated with IL-1β plus TIMP-1 (50 ng/ml) generated 1.64 ± 0.05 μmoles nitrite, which was marginally less than IL-1β–treated islets but not statistically different. Islets treated with TIMP-1 alone did not show any difference in NO production when compared with control islets (1.16 ± 0.05 μmoles · 25 islets^{-1} · 48 h^{-1}). Because the functional significance of this small decrease in NO production in IL-1β plus TIMP-1–treated islets was not clear, we performed Northern blot experiments to further elucidate whether TIMP-1–mediated cytoprotection involves iNOS gene expression.

Northern blot analysis of iNOS mRNA. Northern blot analysis was performed on total RNA extracted from INS-1 cells subjected to cytokines plus or minus TIMP-1. Two independent experiments were performed for each condition, and both demonstrated similar results. As shown in Fig. 8*A*, untreated INS-1 cells (*lane 1*) and INS-1 cells treated with 50 ng/ml TIMP-1 (*lane 2*) had undetectable iNOS gene expression (iNOS-to-GAPDH ratio = 1). A high-dose combination of cytokines (IL-1β 5 ng/ml + TNF-α 30 ng/ml + IFN-γ 30 ng/ml) induced a 4.9-fold increase in iNOS expression (*lane 5*). When TIMP-1 (50 ng/ml) was added to the high-dose cytokine mixture, no decrease in iNOS gene expression was observed (iNOS-to-GAPDH ratio = 4.6) (*lane 6*). We also evaluated the effect of IL-1β alone on iNOS gene expression; as shown in Fig. 8*B*., IL-1β (0.5, 1.0, and 5.0 ng/ml) induced a dose-dependent increase in the iNOS-to-GAPDH ratio of 3.0, 8.7, and 13.9, respectively, compared with untreated INS-1 cells. Once again, TIMP-1 treatment (50 ng/ml) did not alter the iNOS-to-GAPDH ratio when compared with the respective experiment (e.g., for IL-1β 0.5 ng/ml, the ratio was 3.0, and for IL-1β 0.5 ng/ml + TIMP-1 50 ng/ml, the ratio was 2.4). The relatively higher iNOS-to-GAPDH ratios seen in Fig. 8*B* do not reflect higher levels of iNOS induction by IL-1β alone compared with that of the high-dose cytokine mixture. Rather, the higher ratios reflect a lower background density reading obtained in the untreated INS-1 cells in the IL-1β experiment. Therefore, TIMP-1–mediated protection of INS-1 cells from cytokine-induced cell death and dysfunction does not involve iNOS gene expression and consequent NO production.

TIMP-1 inhibits cytokine-mediated activation of NF-κB. We used an NF-κB reporter construct linked to the luciferase gene to evaluate the effect of cytokines plus or minus TIMP-1 on NF-κB activation. Because this promoter construct contains two NF-κB binding sites, the intensity of luciferase activity in this system was directly related to NF-κB activation and translocation into the nucleus. Cytokine-stimulated INS-1 cells transiently transfected with reporter gene demonstrated 4,602 ± 271 fluorescent units,

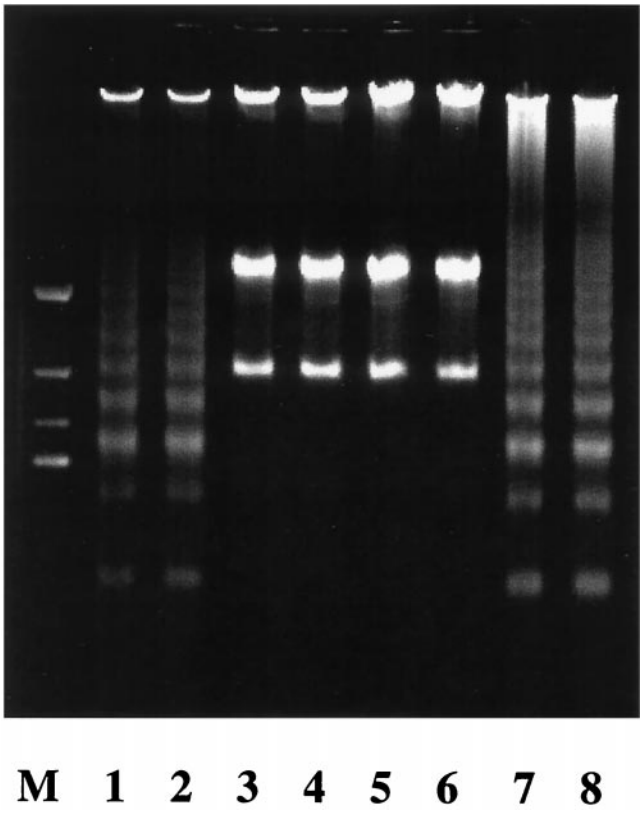

FIG. 2. DNA-laddering experiments with INS-1 cells. INS-1 cells were treated with cytokines plus or minus TIMP-1 and total DNA was isolated thereafter. *Lanes 1* and *2* demonstrate classical DNA laddering when INS-1 cells are treated with cytokines alone. Cytokines plus TIMP-1 (50 ng/ml)–treated cells (*lanes 3* and *4*) show complete inhibition of DNA laddering. *Lanes 5* and *6* show untreated control INS-1 cells, whereas *lanes 7* and *8* show EGTA-treated INS-1 cells (positive control). *Lane M* designates molecular-weight markers. The strong bands seen in *lanes 3–6* represent two heterogeneous DNA populations confirmed by flow cytometry to be expressed in healthy INS-1 cells.

whereas control and cytokine plus TIMP-1 (50 ng/ml)–treated INS-1 cells demonstrated 1,095 ± 87 fluorescent units and 1,071 ± 117 fluorescent units, respectively ($P <$ 0.0001 vs. cytokine-treated INS-1 cells) (Fig. 9). INS-1 cells treated with TIMP-1 alone showed luciferase activity similar to control untreated cells (1,032 ± 76 fluorescent units.) Therefore, TIMP-1 prevented cytokine-induced activation of NF-κB in INS-1 cells.

DISCUSSION

We used a high-dose cytokine model to demonstrate that TIMP-1 prevents apoptosis in pancreatic islets and β-cells. However, we do not know the full spectrum of intracellular events regulated by TIMP-1. Our data demonstrates that TIMP-1 inhibits cytokine-induced activation of the transcription factor NF-κB. Stressful stimuli increase NF-κB activity, resulting in transcription of genes that govern cell fate. Therefore, inhibition of NF-κB activity may reflect the antiapoptotic state induced by TIMP-1 when cytokines are present. Alternatively, TIMP-1–induced inhibition of NF-κB activity may prevent transcription of NF-κB–dependent proapoptotic genes.

However, TIMP-1 does not alter cytokine-induced iNOS gene transcription, as demonstrated by Northern blot analysis. In addition, TIMP-1 appears to have a minimal effect on cytokine-induced NO production in pancreatic β-cells. These results appear to conflict with our NF-κB data because the iNOS promoter contains several NF-κB binding sites and presumably depends on NF-κB binding to initiate gene transcription (22). Studies in rat islets have demonstrated that tyrosine kinase activation precedes NF-κB activation and that NF-κB plays a critical role in iNOS gene transcription (31). Whereas rat islets require only IL-1β to activate iNOS gene transcription, human islets require three cytokines (IL-1β, TNF-α, and IFN-γ). Therefore, additional transcription factors may be necessary for iNOS gene transcription in human islets (32). Because TIMP-1 inhibits cytokine-induced activation of NF-κB, it should be obligatory that TIMP-1 also inhibits iNOS gene transcription. In fact, our data demonstrates quite the opposite result.

Two published studies help to clarify this apparent contradiction by demonstrating that in certain cell types, NF-κB activation is not required for iNOS gene transcription. One study evaluated a similar cytokine mix (IL-1β 50 U/ml, IFN-γ 100 U/ml, and TNF-α 10 ng/ml) on iNOS gene transcription in human epithelial DLD-1 cells (33). Cytokine-induced iNOS gene transcription was inhibited with a number of tyrosine kinase inhibitors, including the Janus kinase (JAK)-2 inhibitor tyrphostin B42, whereas a variety of NF-κB inhibitors (dexamethasone, 3,4-dichloroisocoumarin, panepoxydone, and pyrrolidine dithiocarbamate) did not affect iNOS gene transcription. A second study

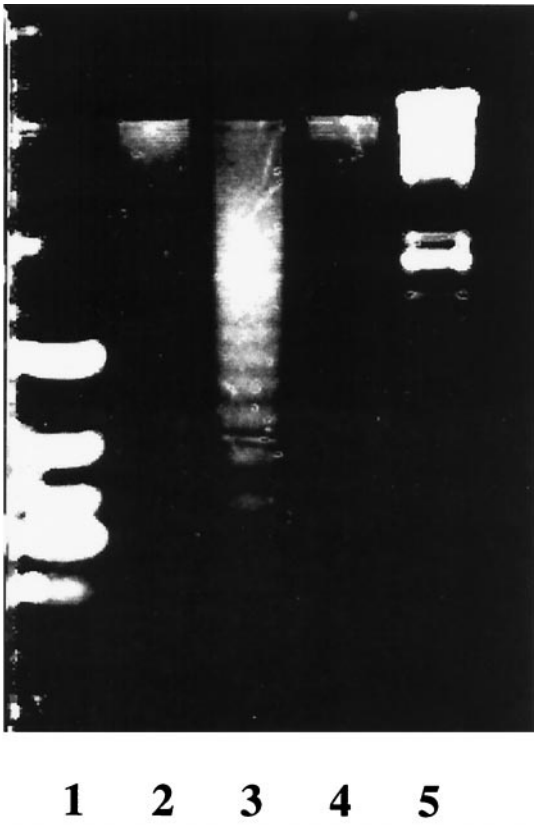

FIG. 3. DNA-laddering experiments with rat islets. Approximately 200 rat pancreatic islets per experiment were treated with cytokines plus or minus TIMP-1, and DNA laddering was performed. *Lanes 1* and *5* show low- and high-molecular-weight DNA markers, respectively. *Lane 2* shows no DNA laddering in untreated rat islets, whereas *lane 3* shows classical DNA laddering in cytokine-treated rat islets. TIMP-1 protected rat islets from cytokine-induced DNA laddering, as shown in *lane 4*.

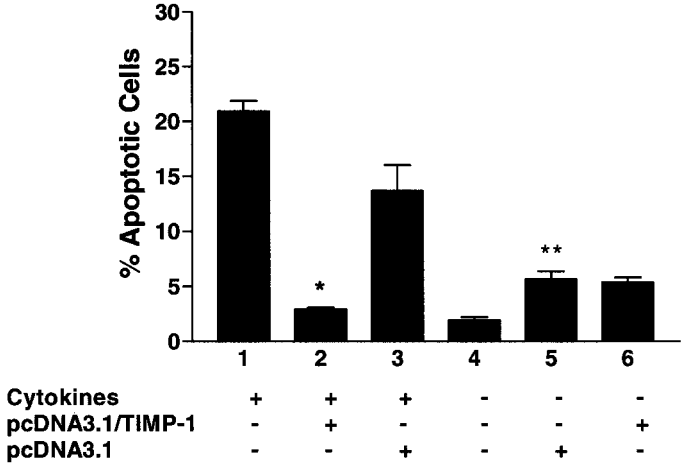

FIG. 4. Flow cytometry on INS-1 cell transfected with pcDNA3.1/TIMP-1 plasmid. Cytokine treatment induced significant apoptosis in nontransfected INS-1 cells (*lane 1*), whereas INS-1 cells transfected with pcDNA3.1/TIMP-1 showed little apoptosis when exposed to the same cytokine mixture (*lane 2*). Cytokine-treated INS-1 cells transfected with the pcDNA3.1 vector alone showed significant apoptosis (*lane 3*), whereas the untreated nontransfected INS-1 cells showed a low level of apoptosis (*lane 4*). In addition, untreated INS-1 cells transfected with either pcDNA3.1 plasmid (*lane 5*) or pcDNA3.1/TIMP-1 plasmid (*lane 6*) showed low levels of apoptosis similar to those of untreated nontransfected INS-1 cells. Each experiment was performed in triplicate and repeated three times for reproducibility. *$P < 0.0001$ vs. *lane 1*; **$P < 0.01$ vs. *lane 3*.

evaluated the effect of AG490 (a JAK-2 inhibitor), JAK-2 antisense oligonucleotides, and a variety of NF-κB inhibitors on cytokine-induced iNOS promoter activity and NF-κB activity (34). Whereas all agents partially prevented cytokine-induced nitrite production, only AG490 and JAK-2 antisense oligonucleotides suppressed iNOS promoter activity without affecting NF-κB. From these studies, we speculate that TIMP-1 acts by inhibiting NF-κB activity but does not alter the JAK-signal transducers and activators of the transcription-signaling pathway (or other obligatory pathways) that might be required for iNOS gene transcription. Therefore, it would be worthwhile to re-evaluate cytokine-induced iNOS gene transcription and NO production in rat and human islets using a high-dose cytokine mix because NF-κB activation may not be an absolute prerequisite in this model.

In the present study, we did not attempt to identify critical anti-apoptotic factors that are induced by TIMP-1.

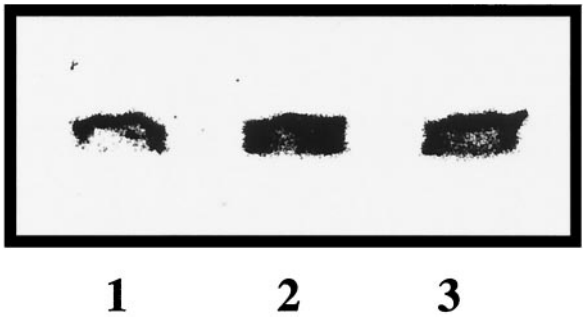

FIG. 5. Western immunoblot of protein extracts from control INS-1 cells (*lane 1*) and transiently transfected INS-1 cells (*lanes 2* and *3*). TIMP-1–transfected cells demonstrated a doublet at molecular weight ~30 kDa and a two- to threefold increase in TIMP-1 protein, whereas nontransfected cells demonstrated a low level of endogenous TIMP-1 protein.

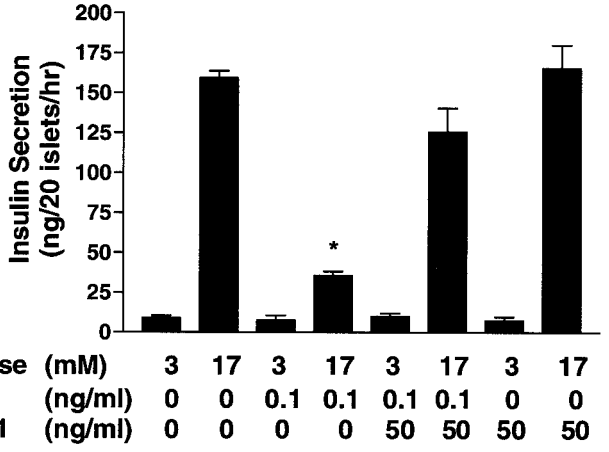

FIG. 6. GSIS in rat islets treated with IL-1β (0.1 ng/ml) plus or minus TIMP-1 (50 ng/ml). Untreated rat islets demonstrated a ~12-fold increase in insulin secretion with 17 mmol/l glucose, whereas IL-1β–treated islets had only a ~2.5-fold increase. Islets treated with IL-1β plus TIMP-1 showed restoration of GSIS to 75% of the control value, whereas islets treated with TIMP-1 alone did not show any alteration in GSIS compared with control islets. Each experiment was performed three to four times and repeated three times for reproducibility. *$P < 0.006$ vs. 17 mmol/l control

Nevertheless, it is reasonable to speculate that, as in Burkitt's lymphoma cells (19), TIMP-1 upregulates Bcl-x(L) in pancreatic β-cells. Recently, Zhou et al. (35) revealed that Bcl-x(L) overexpression in pancreatic β-cells prevented cell death but impaired insulin secretion. Of note, transgenic mice expressing a two- to threefold increase in Bcl-x(L) in their pancreatic β-cells did not display glucose intolerance as did mice expressing a 10-fold increase. In our studies, we observed that higher doses of TIMP-1 (100 ng/ml) led to a 2- to 2.5-fold reduction in GSIS in rat islets (control 94.6 ± 6.7 ng insulin · h^{-1} · 20 $islets^{-1}$ [$n = 4$] vs. TIMP-1–treated 45.1 ± 6.6 ng insulin · h^{-1} · 20 $islets^{-1}$ [$n = 4$]; $P < 0.002$). However, GSIS in cytokine plus TIMP-1 (100 ng/ml)–treated islets was still significantly better than in islets treated with IL-1β alone (45.1 ± 6.6 ng insulin · h^{-1} ·

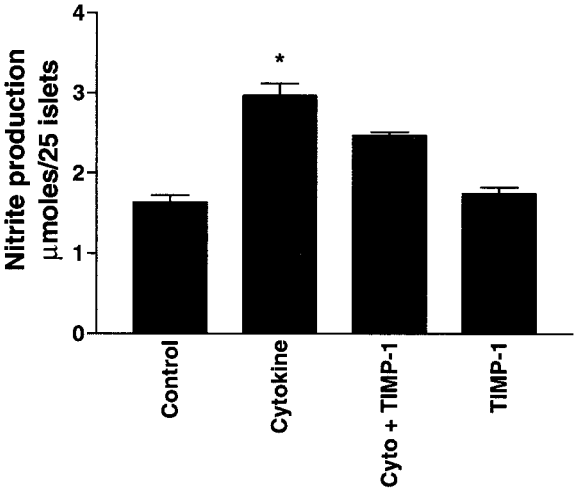

FIG. 7. NO production in rat islets treated with IL-1β plus or minus TIMP-1. Nitrite production was measured in rat islets exposed to IL-1β (0.1 ng/ml) plus or minus TIMP-1 (50 ng/ml) for 48 h. NO was unaffected by TIMP-1 alone, whereas IL-1β plus TIMP-1 caused a small decrease in NO production when compared with IL-1β alone; although not statistically significant. Each experiment was performed in triplicate and repeated twice for reproducibility. *$P = 0.0006$ vs. control.

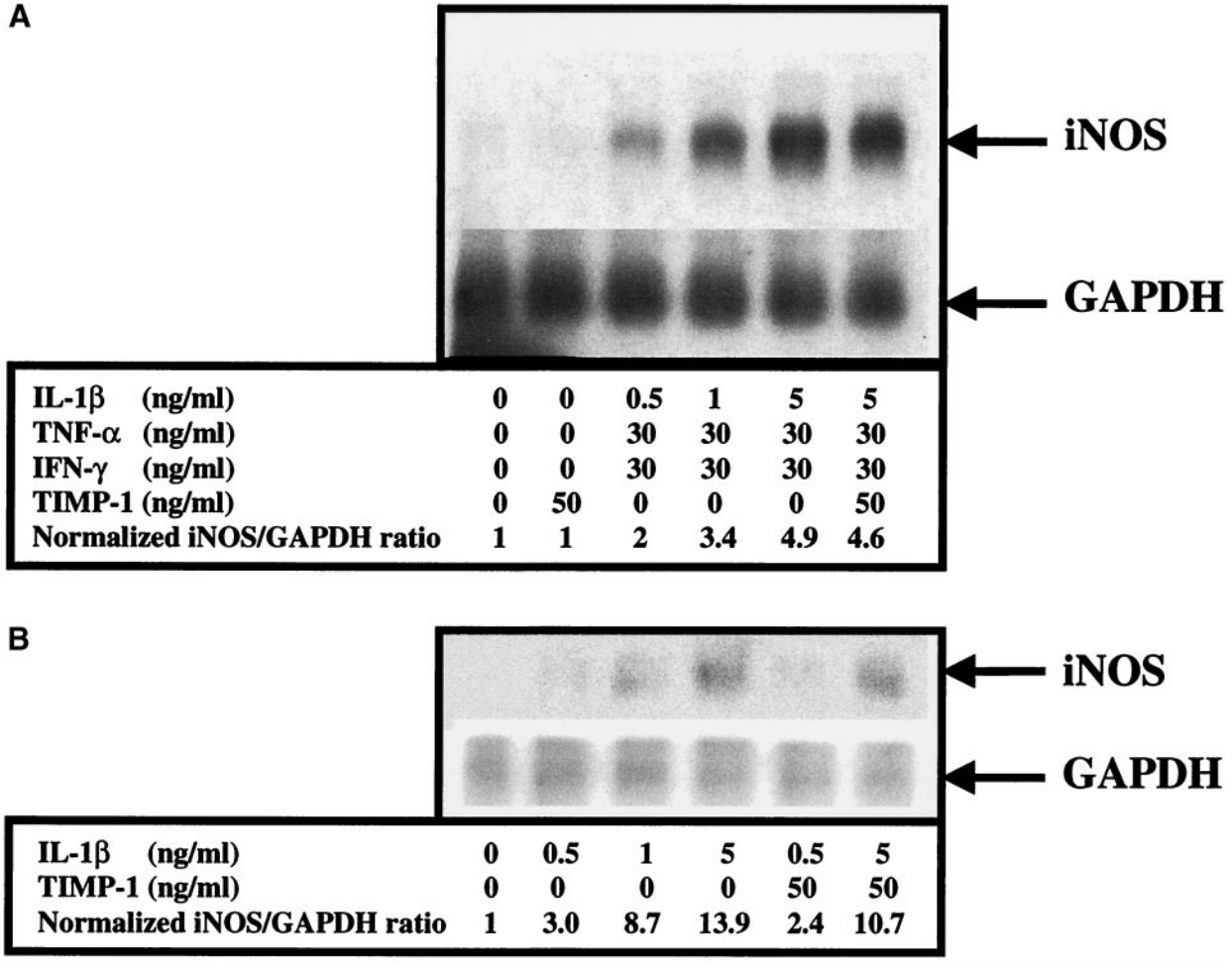

FIG. 8. Northern blot analysis of iNOS mRNA isolated from INS-1 cells. *A*: A high-dose combination of cytokines plus or minus TIMP-1 was used to induce iNOS expression. *B*: IL-1β alone plus or minus TIMP-1 was used to induce iNOS expression. Cytokines induced a significant increase in iNOS mRNA expression in a dose-dependent manner. TIMP-1 addition did not effect iNOS mRNA expression in either experiment.

20 islets^{-1} [$n = 4$] vs. 14.4 ± 0.7 ng insulin · h^{-1} · 20 islets^{-1} [$n = 4$]; $P = 0.001$). Clearly, an optimal dose of Bcl-x(L) or TIMP-1 gene delivered to pancreatic β-cells is necessary to achieve the desired cytoprotective effect without impairing insulin secretion.

The anti-apoptotic effect of TIMP-1 appears to be mediated by a membrane-bound receptor because anti–TIMP-1 antibodies were shown to reverse this effect (19). Moreover, Ritter et al. (36) used time-lapsed confocal microscopy to demonstrate that TIMP-1/EGFP fusion protein binds to the surface of MCF-7 cells and later localizes in the cytosol and nucleus. These studies indicate that TIMP-1 may bind to a cell surface receptor and that the receptor-ligand complex mediates downstream signaling events. Future studies will be necessary to identify the regulatory elements involved in the anti-apoptotic effects of TIMP-1.

Currently, three studies have addressed the role of MMPs and TIMPs in pancreatic islets. Miralles et al. (37) demonstrated that MMP-2 is critical in rat-pancreatic islet development and is activated between embryological days 17 and 19. They found that transforming growth factor (TGF)-β is responsible for islet morphogenesis by regulating MMP-2 expression and that a pan-specific TGF-β–neutralizing antibody could prevent islet development and inhibit MMP-2 expression. Tomita and Iwata (38) performed immunohistochemistry on normal human islets and islet cell tumors taken from patients who underwent pancreatic resection for pancreatic tumors. They observed that nor-

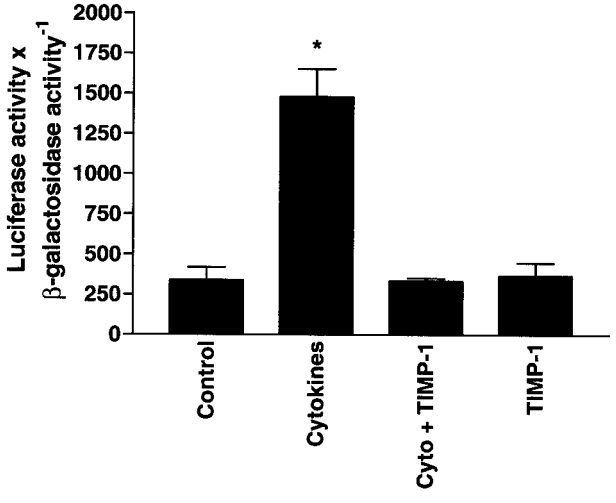

FIG. 9. Luciferase assay for NF-κB activity. INS-1 cells were transiently transfected with a plasmid containing an IL-6 promoter with two NF-κB binding sites. Cytokine stimulation increased NF-κB activity fourfold over control levels. TIMP-1 treatment totally prevented cytokine-induced NF-κB activation. Each experiment was done in triplicate and repeated twice for reproducibility. *$P < 0.0001$ vs. all groups.

mal islets had more intense staining of MMP-2, MMP-9, TIMP-1, and TIMP-2 than islet tumors and that all cell types demonstrated MMP/TIMP staining. Barro et al. (39) evaluated the effect of cytokines on MMP/TIMP expression in rat pancreatic islets. Freshly isolated rat islets were exposed to IL-1β (1 ng/ml) for up to 24 h, at which time reverse transcriptase–PCR for MMPs and TIMPs was performed. Whereas no induction of MMP-2, MMP-9, or TIMP-1 was demonstrated with IL-1β, the authors did find detectable levels of MMP-9 and TIMP-1 after islet isolation; this suggests that the isolation procedure may have stressed the islet. No additional experiments were performed using combinations of cytokines, such as IL-1β, TNF-α, and IFN-γ. Each of these studies demonstrated that pancreatic islets contain detectable amounts of MMPs and TIMPs. Clearly, MMPs play a role in the ontogeny of pancreatic islets, but little is known about whether MMPs and TIMPs regulate the immune response against pancreatic β-cells in type 1 diabetes and islet transplantation rejection.

In summary, we have identified a gene that prevents cytokine-induced islet damage and blocks lymphocyte trafficking to inflammatory sites. Insofar as Th1 lymphocytes release cytotoxic cytokines during the immune response in type 1 diabetes and pancreatic islet transplantation rejection, TIMP-1 may be an ideal gene for protecting pancreatic islets from these insults.

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