

Contribution of Ductal Cells to Cytokine Responses by Human Pancreatic Islets

Dejan Pavlovic, Meng-Chi Chen, Luc Bouwens, Décio L. Eizirik, and Daniel Pipeleers

In type 1 diabetes, autoimmune destruction of pancreatic β -cells has been attributed to cytokines released from infiltrating immunocytes. Exposure of isolated islets to cytokines leads to nitric oxide (NO) production, which can damage β -cells. Because ductal cells are closely associated with human β -cells, we examined whether they can contribute to this process. Isolated human ductal cells were cultured for 48 h with various cytokines. The combination of interleukin-1 β (IL-1 β) plus interferon- γ (IFN- γ) increased nitric oxide production 12-fold while stimulating mRNA expression of inducible nitric oxide synthase (iNOS). In this condition, 10–20% of cells positive for the cytokeratin-19 duct marker also stained positive for iNOS protein, whereas no positive cells were found in control preparations. Comparison of the magnitude of iNOS mRNA expression and nitric oxide production in these cells with that in isolated human islets suggests that >50% of total islet nitric oxide production might originate from associated ductal cells. It is concluded that ductal cells are a potential source of nitric oxide production in human islets infiltrated by cytokine-releasing immunocytes. *Diabetes* 48:29–33, 1999

Autoimmune destruction of pancreatic β -cells in type 1 diabetes may result from direct contacts with islet infiltrating macrophages and T-cells (1) and/or exposure to soluble factors produced by these cells, such as cytokines and free radicals (2,3). Nitric oxide (NO), a product of the enzyme inducible nitric oxide synthase (iNOS), is one of these potentially toxic free radicals (4). Inflammatory cytokines can induce nitric oxide generation by several cell types. In rodent islets, they have been found to stimulate nitric oxide production by β -cells, macrophages, and capillary endothelial cells (5–7). In human islet preparations, they were shown to induce iNOS mRNA expression (8) and nitric oxide production (8,9), but the cell type(s) responsible for this effect has not yet been identified. Compared with isolated rat islet preparations, isolated human islet tissue contains a larger proportion of nonendocrine cells. Culture in serum-free medium results in depletion of acinar cells, endothelial cells, and leukocytes, but not of ductal cells,

which can represent up to 40% of the cells in isolated human islet preparations (10,11). We previously observed that human ductal cells present increased major histocompatibility complex (MHC) class II expression upon exposure to interferon- γ (IFN- γ), suggesting a putative role for these cells in local immune reactions such as those occurring during the insulinitis process (12). The present study investigates whether human ductal cells can also become a source for nitric oxide production in the vicinity of islet endocrine cells.

RESEARCH DESIGN AND METHODS

Cell isolation and culture. Human pancreatic islet and ductal cells were isolated from pancreases obtained from adult heart-beating organ donors. The organs were procured by European hospitals affiliated with the Eurotransplant Foundation (Leiden, the Netherlands) and with β -Cell Transplant, a multicenter program on β -cell transplantation in diabetes. They were processed at the Central Unit in Brussels, as previously described (10–12). Mean donor age was 42 ± 3 years (mean \pm SE; range 18–63 years). Collagenase digests were separated by Ficoll gradient purification into an islet fraction and a ductal cell fraction. After culture in serum-free medium for at least 3 days, one preparation was enriched in endocrine cells and the other in ductal cells (Table 1). In one set of experiments, the endocrine-enriched fraction was dispersed by trypsin and submitted to fluorescence-activated cell sorting to isolate endocrine cells of >70% purity (13). The culture medium consisted of HAM's F-10 with 6 mmol glucose, 1% bovine serum albumin (Boehringer Mannheim, Mannheim, Germany), 0.1 mg/ml streptomycin (Continental Pharma, Puteaux, Belgium), 125 U/ml penicillin (Laboratoires Diamant, Brussels, Belgium) and 2 mmol L-glutamine (GIBCO, Paisley, Scotland) (12). In some experiments, HAM's F-10 medium was supplemented with 1 mmol L-N-methyl-arginine (Sigma, Milwaukee, WI). Cultures for medium nitrite determination and for hormone or cytokeratin immunocytochemistry were carried out in suspension dishes of 3 cm in diameter (Nunc, Naperville, IL), with 2×10^6 to 6×10^6 cells/2 ml. For iNOS immunocytochemical staining, permanox two-well slide-chambers (Nunc) were used with 0.5×10^6 to 1×10^6 cells/2 ml of medium supplemented with 10% fetal calf serum. Addition of serum was needed for cell attachment and spreading, which were required to observe positive staining with the iNOS antibody. The effect of cytokines was examined after culture for 48 h in the presence of human IFN- γ (1,000 U/ml, 97% pure, 47.5 U/ng; Genzyme, Cambridge, MA), human interleukin-1 β (IL-1 β) (50 U/ml, 95% pure, 200 U/ng; Genzyme), and murine tumor necrosis factor- α (TNF- α) (1,000 U/ml, 98% pure, 220 U/ng; Innogenetics, Gent, Belgium), either alone or in combination. At the end of this culture period, supernatants were retrieved for nitrite determination by the Griess reaction (14) and pellets were collected for DNA assay or for RNA extraction.

Human standardized dermal fibroblasts were obtained from PromoCell (Heidelberg, Germany). They were cultured in PromoCell's fibroblast growth medium in three passages until confluency was reached. Exposure to cytokines was performed under the same culture conditions as those for islet and ductal cell preparations.

Reverse transcription-polymerase chain reaction analysis of iNOS gene expression. RNA was extracted with RNeasy Mini kit (QIAGEN, Hilden, Germany), and reverse transcription (RT)-polymerase chain reaction (PCR) was performed with the GeneAmp RNA PCR kit (Perkin-Elmer, Norwalk, CT). For RT, the 10 μ l reaction mixture contained 0.2 μ g RNA, 10 mmol Tris-HCl (pH 8.3), 50 mmol KCl, 5 mmol MgCl₂, 1 mmol of each deoxynucleoside triphosphate, 2.5 μ mol/l random hexamer primers, 10 U RNase inhibitor, and 25 U M-MLV reverse transcriptase. The reaction mixture was incubated at room temperature for 10 min, then at 42°C for 1 h. It was then heated at 99°C for 5 min and immediately chilled on ice.

For PCR, each reaction sample contained 10 μ l cDNA, 10 mmol Tris-HCl (pH 8.3), 50 mmol KCl, 2 mmol MgCl₂, 0.2 mmol of each deoxynucleoside triphosphate, 0.4 μ mol/l of each iNOS primer, and 1.25 U Taq DNA polymerase in a final

From the Diabetes Research Center, Vrije Universiteit Brussel, Brussels, Belgium.

Address correspondence and reprint requests to Daniel Pipeleers, Diabetes Research Center, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium. E-mail: dpip@mebo.vub.ac.be.

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IFN- γ , interferon- γ ; IL, interleukin; iNOS, inducible Nitric oxide synthase; MHC, major histocompatibility complex; OD, optical density; PCR, polymerase chain reaction; RT, reverse transcription; TNF, tumor necrosis factor.

volume of 50 µl. Thermal conditions were as follows: an initial denaturation of 2.5 min at 94°C; 30 cycles, each consisting of denaturation, at 94°C for 45 s; annealing at 58°C for 45 s; and extension at 72°C for 1.5 min. The final PCR cycle was followed by an extension at 72°C for 10 min. In parallel, an aliquot of each cDNA preparation was amplified with GAPDH-specific primers for 25 cycles using a thermal profile similar to iNOS. All PCRs were performed in a Perkin-Elmer 9600 Thermal Cycler. The following primer sequences were used: iNOS-F: 5'-AGAAGTCTCCAGAATCTCTG-3'; iNOS-R: 5'-TGGCCAGATGTTCCCTCT ATT-3'; GAPDH-F: 5'-CATGTTTCGTCATGGGTGTGA-3', and GAPDH-R: 5'-AGT GAGCTCCCGTTTCAGCT-3'. Five microliters of PCR products were separated by electrophoresis in 2% agarose gel, and the ethidium bromide-stained gels were photographed under ultraviolet transillumination. The fragment intensities were scanned by Enhanced Laser Densitometer Ultrosan XL (LKB, Bromma, Sweden) of the negative films and were expressed in arbitrary units of optical density (OD). The OD obtained for iNOS was expressed as a function of the corresponding OD values for GAPDH.

Immunocytochemical studies. After suspension culture, cells were fixed for 4 h in 4% (vol/vol) formaldehyde, embedded in paraffin, sectioned, and exposed to primary antibodies for 16 h at 4°C. The reaction was visualized by peroxidase or alkaline phosphatase, after 30 min exposure at 20°C to a secondary avidin-biotin complexed antibody (15). The cultured cell monolayers of the permanox slide chamber were fixed for 10 min in 4% (vol/vol) formaldehyde. This was followed by 10 min fixation/permeabilization with chilled methanol. The samples were exposed to primary antibodies for 16 h at 4°C and to secondary phycoerythrin- or fluorescein-labeled antibodies for 30 min at 20°C. Antibodies used in this study were mouse anti-cytokeratin-19 (Dako, Glostrup, Denmark), which is used as a ductal cell specific marker (10,11); mouse anti-vimentin (Boehringer Mannheim); mouse anti-CD68 (Dako); guinea pig anti-porcine insulin and rabbit anti-porcine glucagon (a kind gift of C. Van Schravendijk, Diabetes Research Center, VUB, Brussels); mouse anti-factor VIII (Biogenex, San Ramon, CA); rabbit anti-iNOS (N-20) (Santa Cruz Biotechnology, Santa Cruz, CA); and mouse anti-chromogranin A (Biogenex). Negative controls were prepared either by omission of the first antibody, by use of isotype-matched control antibodies, or by appropriate control serum. According to the information from Catherine Chappell-Ybara (Santa Cruz Biotechnology), the NOS2 (N-20) sc-651 antibody was raised against amino acids 9–20 of human NOS2, a sequence with little homology to any other known protein. In Western blots, this antibody recognizes the NOS2 band (approximately 117 kDa) in RAW264.7 whole-cell lysates; binding is blocked with the sc-651 peptide. The immunostained samples were analyzed in light and fluorescence microscopy. Fluorescence images were recorded by chilled CCD camera (Hamamatsu, Hamamatsu, Japan).

RESULTS

Nitric oxide production and iNOS expression by human ductal cell preparations. Exposure of human ductal cell preparations for 48 h to the cytokines IL-1β, TNF-α, or IFN-γ did not increase medium nitrite accumulation above control levels (Fig. 1). Increased medium nitrite levels were measured when cells were exposed to a combination of IL-1β plus IFN-γ, but not of TNF-α plus IL-1β or IFN-γ (Fig. 1). This cytokine-induced nitrite production did not occur in an arginine-free medium or in the presence of L-N-methyl-arginine (0.5 mmol/l) (data not shown), which is known to inhibit iNOS activity. The combination of IL-1β and IFN-γ was found to increase expression of iNOS mRNA (Fig. 2). TNF-α did not

potentiate nitrite production or iNOS mRNA expression induced by IL-1β plus IFN-γ (Figs. 1 and 2).

Comparison between nitrite production and iNOS mRNA expression by ductal and islet cell preparations.

To assess to what extent ductal cells contribute to nitrite production by human islet cell preparations, we compared nitric oxide production after cytokine exposure in ductal and islet cell preparations from the same donor. When medium nitrite levels were expressed per microgram DNA, they were found to be increased 18-fold in both islet and ductal cell preparations exposed to IL-1β plus IFN-γ. Both preparations thus contain cells in which nitric oxide production can be stimulated to the same extent during exposure to cytokines. In view of the potentially damaging effect of high nitric oxide concentrations, it is more relevant to compare the respective production rates in absolute terms. The nitrite levels of the medium were found to be 60% higher in ductal cell than in islet cell preparations (Table 1). Considering the percentage of ductal cells present in human islet cell preparations (Table 1) and assuming that these contaminating cells exhibit the same rates of nitric oxide production as pure ductal cells, it appears that at least 50% of the levels measured in human islet preparations can be generated by ductal cells.

A similar conclusion was reached after comparing iNOS mRNA expression in purified human endocrine cells (containing <20% ductal cells) with that observed in ductal cells (containing >90% ductal cells) obtained from the same donor (n = 3). Both preparations expressed iNOS mRNA, but expression in the endocrine preparation was 61 ± 8% of that observed in ductal cells (the OD of iNOS relative to the OD of GAPDH).

Identification of iNOS protein in ductal cell preparations.

Double immunocytochemical staining with antibodies for iNOS and for the ductal cell marker cytokeratin-19 indicated the presence of double-positive cells in ductal cell preparations (Fig. 3). These cells were only noticed after exposure to IL-1β plus IFN-γ; they represented 10–20% of the cells positive for cytokeratin-19. In addition, a small number (<10%) of iNOS-positive cells were negative for markers for ductal cells (cytokeratin-19), endocrine cells (chromogranin), endothelial cells (factor VIII), macrophages (CD68), and fibroblasts/macrophages (vimentin). When immunostaining was conducted in islet cell preparations, we noticed that iNOS expression was mainly associated with cells expressing cytokeratin-19. A few iNOS-positive cells were identified within the cellular aggregates that appeared positive for the endocrine cell marker chromogranin (data not shown). However, failure to form monolayers from

TABLE 1
Nitrite production by human islet and ductal cell preparations

Isolated fraction	Cell composition (percentage of cells positive for:)			Nitrite content of medium (pmol · l ⁻¹ · µg ⁻¹ DNA · h ⁻¹)	
	Insulin	Glucagon	Cytokeratin-19	Control	IL-1β + IFN-γ
Islet cells	51 ± 4	17 ± 4	32 ± 4	0.7 ± 0.1	11.3 ± 2.8
Ductal cells	1	<1	98 ± 1	1.5 ± 0.3	18.0 ± 3.3

Data are means ± SE of six independent experiments, each performed on material from a different donor. Pancreatic islet and ductal cell preparations obtained from the same donor were cultured in parallel under the same conditions for 48 h with and without IL-1β (50 U/ml) plus IFN-γ (1,000 U/ml). Culture medium was assayed for the nitrite content.

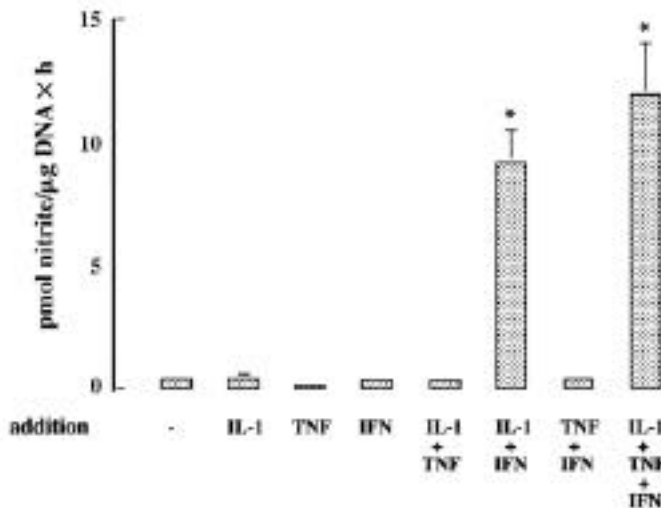


FIG. 1. Ductal cell preparations were cultured for 48 h in the absence or presence of IL-1 β (50 U/ml), TNF- α (1,000 U/ml), and IFN- γ (1,000 U/ml), alone or in combination. Medium was collected for determination of its nitrite content. The results are means \pm SE of five independent observations ($P < 0.0001$ vs. control, analysis of variance).

endocrine aggregates makes it impossible to identify the iNOS-positive cell types and their respective percentages. Confocal microscopy might be useful in this respect and could indicate whether some of these iNOS-positive cells are insulin positive. The same technique should examine whether iNOS-positive, cytokeratin-19-positive cells exhibit any insulin-immunoreactive material; we have been unable, so far, to identify such cells using conventional techniques.

Endothelial cells and macrophages are unlikely participants, because they are virtually absent in our human islet and ductal cell preparations (16). Occasional fibroblasts (<3%) were identified. We therefore examined whether human fibroblasts could contribute to nitrite accumulation. After culture under the same conditions as ductal and islet cell preparations,

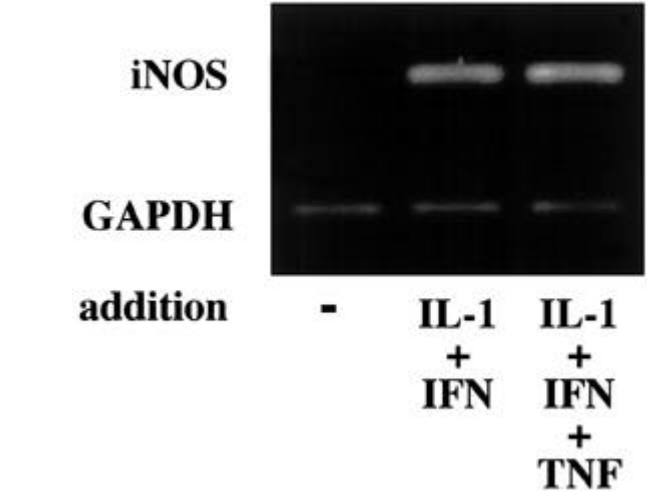


FIG. 2. RT-PCR analysis of human iNOS mRNA expression by ductal cells after 48 h culture in the absence or presence of IL-1 β plus IFN γ or IL-1 β plus IFN- γ plus TNF- α (same concentrations as in Fig. 1). The cDNA samples were amplified in parallel by GAPDH-specific primers, confirming similar sample loading. The figure is representative of three independent experiments.

normal human fibroblasts did not increase nitrite accumulation after exposure to cytokines (data not shown, $n = 3$).

DISCUSSION

It has been suggested that locally produced nitric oxide may contribute to β -cell dysfunction and damage in type 1 diabetes (4). In vitro human islet cells die when exposed to nitric oxide or peroxynitrite, a toxic radical generated by the reaction between nitric oxide and hydrogen peroxide (4,17,18). There is now in vivo evidence that both nitric oxide and peroxynitrite are produced in and around β -cells during insulinitis in NOD mice (19). Production of nitric oxide can be induced by cytokines in isolated rodent islets (4). This effect has been attributed to iNOS expression in β -cells

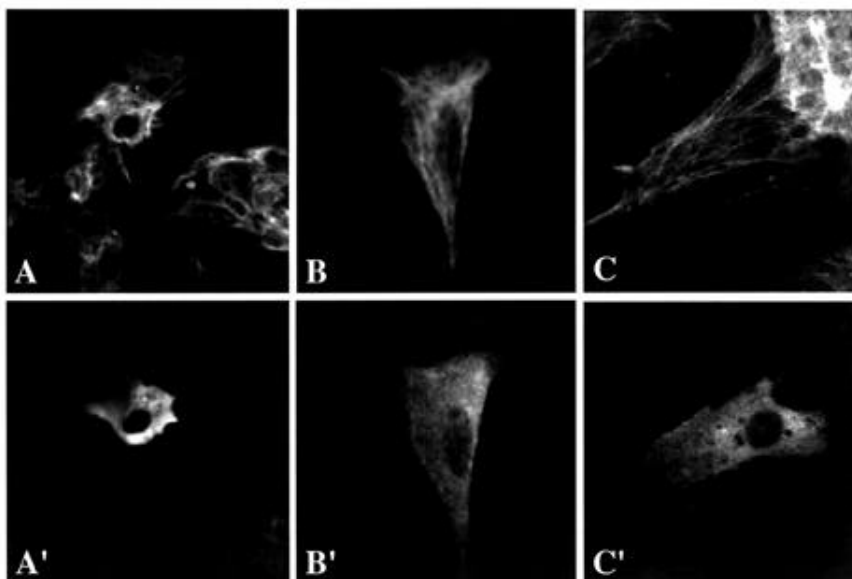


FIG. 3. Double immunocytochemistry for the ductal cell-specific marker cytokeratin-19 (A, B, C) and iNOS (A', B', C') in a human ductal cell preparation after 48 h exposure to IL-1 β plus IFN- γ (same concentrations as in Fig. 1). The figure is representative of five independent experiments. Cytokeratin positivity appears as filaments; iNOS positivity, as a diffuse paranuclear staining. Original magnification $\times 500$ for A, A' and $\times 1,000$ for B, B', C, C'.

(4,5), in islet endothelial cells (7), and/or in activated macrophages (6). In human pancreatic islet preparations, nitric oxide can also be produced during cytokine exposure (8,9,20), but it appears to be nonessential for the functional or structural consequences at the level of the β -cells (8,13,20). It still remains unclear which cell type(s) is primarily responsible for the nitric oxide production in human islets. Isolated human islets contain a larger proportion of nonendocrine cells than do isolated rat islets (10,11). After culture, up to 40% of the islet cells correspond to ductal cells, which are identified by their cytokeratin-19 marker (10,11, present data). In the human pancreas, cytokeratin-19-positive ductal cells are often closely associated to the insulin-positive β -cells (21). Because human ductal cells were previously found to become positive for MHC class II after exposure to the cytokine IFN- γ (12), we examined whether this or other cytokines also induced nitric oxide production from this cell type. It was found that human ductal cells exposed to IL-1 β plus IFN- γ express iNOS at both the mRNA and the protein level and produce nitric oxide as determined by nitrite accumulation in the medium.

Immunocytochemical analysis indicated that only 10–20% of the ductal cells became iNOS positive. In previous studies on rat β -cells (4), neurons (22), megakaryocytes (23), and dendritic cells (24), cytokines were consistently found to induce iNOS positivity in only a subpopulation of cells. For technical reasons, we are so far unable to identify iNOS positivity in human β -cells that have been exposed to cytokines. However, a recent study using an antibody for human iNOS that is not commercially available indicated that human β -cells stain positively for iNOS after exposure to cytokines (25). This, and the observation that cytokines induce iNOS mRNA expression and nitrite production by human preparations enriched in endocrine cells, is certainly compatible with a contribution by the β -cells but does not exclude a contribution by other cells. According to the present data, ductal cells represent a major source for nitric oxide production in human islet preparations, if indeed islet ductal cells exhibit a similar responsiveness to cytokines as those observed in isolated ductal cell preparations. Ductal cells are routinely observed at relatively high percentages (30–40%) in our isolated human islet preparations. In other laboratories, this percentage appears markedly lower as judged from the reported high percentage of endocrine cells detected by the dithizone method (26,27). However, adequate determination of the frequency of cell types requires the use of cell-specific markers, such as insulin for the β -cells and cytokeratin-19 for the ductal cells (12,21). In doing so, we have noticed that nonendocrine ductal cells should be considered mediators of cytokine effects in the islet region.

The present findings further underline the potential role of ductal cells in the development of type 1 diabetes. Their location at the periphery of pancreatic islets might make them a major target for cytokines that are released from the peripherally located immunocytes that are often present in the early stages of insulinitis (28). If these human infiltrating cells express IL-1 β and IFN- γ as in rodent models of insulinitis (29), it can be envisaged that they induce expression of both MHC class II and iNOS in islet-associated ductal cells. They may, independent of each other, result in antigen presentation and in nitric oxide production and hence amplify the immune-mediated effects and reactions. Depending on the site and intensity of nitric oxide production, adjacent β -cells might

become dysregulated or damaged (4,9,17–19), or putative β -cell precursor cells might lose their potential to replicate or differentiate into β -cells (15).

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Author Queries (please see Q in margin and underlined text)

Q1: Units in Table 1 correct?

Q2: Fig. 1—Please explain asterisks in legend.