Distinction Between Interleukin-1–Induced Necrosis and Apoptosis of Islet Cells

Anne Hoorens, Geert Stangé, Dejan Pavlovic, and Daniel Pipeleers

Interleukin (IL)-1β is known to cause β-cell death in isolated rat islets. This effect has been attributed to induction of nitric oxide (NO) synthase in β-cells and subsequent generation of toxic NO levels; it was not observed, however, in dispersed rat β-cells. The present study demonstrates that IL-1β induces NO-dependent necrosis in rat β-cells cultured for 3 days at high cell density or in cell aggregates but not as single cells. Its cytotoxic effect is not explained by higher NO production rates but might result from higher intercellular NO concentrations in statically cultured cell preparations with cell-to-cell contacts; nitrite levels in collected culture medium are not a reliable index for these intercellular concentrations. Absence of IL-1–induced necrosis in rat α-cells or in human β-cells is attributed to the cytokine’s failure to generate NO in these preparations, not to their reduced sensitivity to NO: the NO donor GEA 3162 (15 min, 50–100 μmol/l) exerts a comparable necrotic effect in rat and human α- or β-cells. In preparations in which IL-1β does not cause β-cell necrosis, its combination with γ-interferon (IFN-γ) results in NO-independent apoptosis, starting after 3 days and increasing with the duration of exposure. Because IFN-γ alone was apoptotic for rat α-cells, it is proposed that IL-1β can make β-cells susceptible to this effect, conceivably through altering their phenotype. It is concluded that IL-1β can cause NO-dependent necrosis or NO-independent apoptosis of islet cells, depending on the species and on the environmental conditions. The experiments in isolated human β-cell preparations suggest that these cells may preferentially undergo apoptosis when exposed to IL-1β plus IFN-γ unless neighboring non–β-cells produce toxic NO levels. *Diabetes* 50:551–557, 2001

**RESEARCH DESIGN AND METHODS**

**Preparation and culture of rat β- and α-cells and islets.** Pancreatic islets were isolated from adult male Wistar rats, as described previously (26). Freshly isolated islets were dissociated in calcium-free medium and purified into single β-cells (>92% β-cells) and α-cells (>75% α-cells) by autofluores-
ence-activated sorting (26). The purified β-cell preparation contained <5% α-cells, <5% PP cells, <5% δ-cells, and <5% nondendritic islet cells and contaminating exocrine cells; the α-cell preparation contains 10% β-cells, 5% PP cells, <5% δ-cells, and <5% nondendritic islet cells and contaminating exocrine cells (26). Isolated single cells were cultured in polylysine-coated microtiter cups (3 × 10^4, 24 × 10^4, or 48 × 10^4 cells/cup in 200 l̅ μl medium) (27). Isolated β-cells were also reaggregated in a rotary shaking incubator (28) before further culture in polylysine-coated multiwells (15 × 10^6 cells/well in 1 ml medium); isolated islets were cultured in the same conditions (15 islets/well in 1 ml medium). Culture was conducted in serum-free medium that was previously shown to maintain the viability and glucose responsiveness of isolated rat and human β-cells (27,29,30). This medium consists of Ham's F10 (Gibco Laboratories, Grand Island, NY) supplemented with 2 mmol/l L-glutamine, 50 mmol/l β-isobutyl-1-methylxanthine, 0.075 g/l penicillin, 0.1 g/l streptomycin, 10 g/l glucose, and 10 g/l charcoal-treated bovine serum albumin (factor V; radioimmunoassay grade; Sigma, St. Louis, MO). In experiments where cytotoxicity on rat cells (15–21% of all cells), which suggests that it involves cells that were damaged during the isolation procedure and/or nonendocrine contaminating cells that did not survive under the selected culture conditions. Experimental conditions are introduced after this first day and tested for a period of a maximum of 9 days, during which cell death in the control conditions is only 1% per day. These culture values confirm the ability of our serum-free culture medium to maintain survival of endocrine islet cells during 9 days of culture (27).

Cell death in aggregated cells and islets was evaluated with the same fluorescence methodologies. However, precise quantification of the number of living, apoptotic, and necrotic cells was not possible in this compact tissue. This technical obstacle cannot be overcome by dissociating the aggregates or islets before counting because such a step disrupts cells with damaged membranes and in itself causes cell death (34). We can thus only identify conditions with marked apoptosis and/or necrosis in these preparations.

**Statistical analysis.** Data represent means ± SE of n independent experiments. Each experiment is conducted with cells obtained from different rat or human pancreases. Statistical significance of differences was determined using the Student's paired t test.

## Results

### Apoptosis of single rat and human β-cells. A 9-day exposure of single rat or human β-cells (3 × 10^6 cells/200 μl) to IL-1β up to 50 U/ml was not cytotoxic (Fig. 1). Combination of IL-1β (50 U/ml) plus IFN-γ (1,000 U/ml) induced apoptosis in both preparations, starting after 3 days (apoptosis index 10 ± 1%) and progressively increasing with culture duration (apoptosis index 33 ± 2% after 9 days; Fig. 1). It also caused necrosis in a small percentage of rat β-cells but not in human islet cells; this effect did not increase with the exposure time (14 ± 3 and 13 ± 4% after 6 and 9 days, respectively; P < 0.05 vs. control culture without cytokines, paired t test). Culture with only IFN-γ did not exert any cytotoxic effect (Fig. 1). After 9 days of culture, the percentage of dead cells in the IL-1β plus IFN-γ condition was 69 ± 2 vs. 28 ± 5% in the control for rat β-cells and 56 ± 6% in the IL-1β plus IFN-γ condition vs. 34 ± 6% in the control for human β-cells. All further analysis is carried out on the calculated necrosis and apoptosis index, which facilitates comparisons of the experimental conditions.

The presence of L-methylarginine completely prevented cytokine-induced necrosis of rat β-cells (Fig. 1) but exerted only a partial and transient protection against apoptosis: apoptosis index 4 ± 1, 13 ± 4, and 28 ± 7% after 3, 6, and 9 days of culture, respectively, with IL-1β plus IFN-γ (P < 0.05, <0.05, and >0.05 vs. 10 ± 1, 25 ± 3, and 33 ± 2, respectively, in the absence of L-methylarginine; Fig. 1). In human β-cell preparations, L-methylarginine did not reduce the apoptosis index after 9 days of culture with the cytokine combination (14). The protective effect of L-methylarginine against cytotoxicity induced by exposure of single rat β-cells to IL-1β plus IFN-γ was associated with its prevention of NO accumulation in the medium (medium nitrites in cultures of single rat β-cells exposed to IL-1β plus IFN-γ were 1.3 ± 0.3 and 5.5 ± 0.7 pmol/μl medium in the presence and absence of L-methylarginine).

Nitrite levels were only measured during the first 72 h
because their values are difficult to interpret at later time points in view of the increasing number of dead cells (Fig. 1). These data indicate that both rat and human β-cells can undergo apoptosis after prolonged exposure to IL-1β plus IFN-γ but not to the single cytokines, suggesting involvement of two pathways. This process does not require production of NO but seems accelerated by it (see data on rat β-cells). The cytokine combination can also cause necrosis of rat β-cells, but this effect is completely NO-dependent; this might explain why no necrosis was noticed in human β-cell preparations (Fig. 1) in which the cytokines failed to induce detectable NO levels under the present experimental conditions (<0.5 pmol/μl after 3 days of culture).

**Apoptosis of rat α-cells.** The combination of IL-1β plus IFN-γ was at least equally potent in inducing apoptosis of rat α-cells and rat β-cells but failed to cause necrosis of these cells (apoptosis index in rat α-cells 50 ± 6% and necrosis index 10 ± 3%, P < 0.01 and >0.05, respectively, vs. control condition without cytokines, n = 4). At variance with β-cells, the α-cells also underwent apoptosis when cultured with only IFN-γ (apoptosis index 25 ± 2% and necrosis index 6 ± 3%, P < 0.01 and >0.05, respectively, vs. control condition without cytokines, n = 4). Exposure to IL-1β alone was not cytotoxic for rat α-cells (apoptosis and necrosis index <5%, n = 4). The cytokine-induced apoptosis of α-cells was not prevented by L-methylarginine (apoptosis index 41 ± 5% after exposure to IFN-γ in the presence of L-methylarginine and 52 ± 5% after exposure to IL-1β plus IFN-γ in the presence of L-methylarginine, P < 0.01 vs. control condition without cytokines, n = 4). In none of the α-cell cultures did medium nitrite rise to detectable levels, suggesting independence of NO production (<0.5 pmol/μl after 3 days of culture). This result also indicates that none of the cell types present in this fraction produce any detectable NO levels. Some of these cell types contaminate the purified β-cell preparation, but at lower percentages than in the α-cell preparation; they therefore do not contribute to the NO levels that are measured in the β-cell preparation.

**Necrosis of β-cells cultured at high cell density.** When single rat β-cells were plated at higher densities than the control condition (3 × 10⁴ cells/200 μl), they became subject to IL-1β–induced necrosis but not to apoptosis (Table 1). At 8- and 16-fold higher cell densities, the single cytokine caused a necrosis index of 27 ± 12 and 45 ± 7% after 9 days of culture (Table 1). This cytotoxic effect was not yet apparent after 3 days of culture (necrosis index 5% for the 16-fold higher cell density, P < 0.05 vs. control condition with 3 × 10⁴ cells/200 μl). At the end of the first 3 days, medium nitrite concentration was two- to threefold higher at the higher cell densities. In the presence of L-methylarginine, medium nitrites did not rise above control values, and no necrosis was induced by IL-1β. When medium nitrite accumulation over the first 3 days was expressed as a function of the
respective cell numbers, higher values were found for the low–cell density condition, indicating that the absence of necrosis in this preparation is not due to lower NO production rates (Table 1). During subsequent days, the nitrite accumulation in the medium markedly decreased in the high cell density conditions (data not shown), which is attributed to the increasing proportion of dead cells.

**Necrosis of α- and β-cells in islet cell aggregates.** In reaggregated β-cells or in intact islet tissue, a precise quantification of the percentage of necrotic and apoptotic cells is difficult if not impossible. The currently used fluorescence method can be used to identify outspoken conditions of necrosis or apoptosis in such preparations. It was thus noticed that IL-1β alone caused massive necrosis in reaggregated β-cells; this effect did not occur in the presence of L-methylarginine (Fig. 2). Similar observations were made in cultured islets; electron micrographs indicated that necrosis affected both α- and β-cells (data not shown). The appearance of necrosis in β-cell aggregates and in islets occurred at an earlier time point (after 3 days of culture) than in high-density monolayers (see above). The medium nitrite accumulation during the first 3 days was lower than that in the condition of single β-cells (Table 2). When this nitrite accumulation was expressed as a function of the number of β-cells or of the DNA content, values were also lower than those in the single β-cell condition (Table 2).

**NO-induced necrosis of single rat and human islet cells.** A 15-min exposure of rat and human islet cells to the NO donor GEA 3162 caused necrosis within 24 h (Table 3). Further culture for 5 days did not increase the percentage of necrotic cells. In the three tested cell preparations, the transition from marginal to massive cytotoxicity occurred in a narrow concentration range, i.e., between 25 and 100 μmol/l (Table 3). Within this narrow range, rat α-cells appeared more sensitive than rat β-cells (Table 3). In none of the conditions did the NO donor induce apoptosis.

**DISCUSSION**

Numerous studies have reported that proinflammatory cytokines can be toxic for islet β-cells (2–4). Using different techniques, several laboratories have noticed that cytokines can induce apoptosis in islet cells (14,35–38). A microscopic fluorescence assay allows one to quantify the number of apoptotic cells during culture and to determine the relative importance of apoptosis versus necrosis (25). We have used this method to investigate the effects of IL-1β, which has been a major component in most previous studies. It was found that IL-1β can kill β-cells by necrosis as well as by apoptosis. However, induction of
The reported cell death in IL-1–cultured rat islets (5–10) most likely corresponded with a process of necrosis. Nitrite measurements in collected medium allowed us to identify the NO dependency of this toxicity, but they are not a valid index for the occurrence of this form of cell death because they are not always indicative of the NO levels at the β-cells. Experiments with RINm5F insulinoma cells also indicated that NO production is necessary but not sufficient for a cytokine-induced cell destruction (39). We did not observe a correlation between medium nitrite levels and the occurrence of necrosis in single or aggregated cells. In fact, medium nitrites were lower in reaggregated β-cells and in islets: this probably reflects the functional deficiency that proceeds to necrosis.

Experiments with the NO donor GEA indicated that human β-cells exhibit a susceptibility to NO-induced necrosis comparable to that of rat β-cells. That IL-1β (alone or in combination with IFN-γ) did not induce necrosis in our human β-cell preparation can be attributed to the failure to induce NO production—at least to any detectable levels. We have recently reported that the combination of IL-1β plus IFN-γ can induce iNOS expression and NO production in pancreatic duct cells (40); this non-endocrine cell type is also identified in the purified human β-cell preparation but in numbers that are too low to produce any detectable NO (D.Pa., D.Pi., unpublished data). It is currently unknown whether in other circumstances duct cells can produce sufficient NO to cause necrosis of adjacent β-cells. Studies in freshly isolated human islet preparations have also shown that IL-1β plus IFN-γ can induce iNOS expression in human β-cells (41), which raises the possibility that the state of the β-cells or the nature of the surrounding cells determines their NO production when exposed to cytokines and, hence, their susceptibility to necrosis.

When IL-1β was combined with IFN-γ, both single rat and single human β-cells died by apoptosis starting after 3 days of exposure and progressively increasing with culture time; this process was somewhat delayed when NO production was suppressed by L-methylarginine but was essentially NO-independent. Exposure of β-cells to IL-1β might make them susceptible to apoptosis induction by IFN-γ. IFN-γ alone indeed causes apoptosis in islet α-cells but not in β-cells. We have recently reported that IL-1β alters the phenotype of rat β-cells, causing a reduction in their cell-specific properties (42). This alteration may well be accompanied by a loss in β-cell–specific protection against apoptosis (24), making the cells more susceptible to apoptosis inducers such as IFN-γ.

The present in vitro experiments do not provide information on the effectors and mode of β-cell death in type 1 diabetes. This objective is difficult to achieve with in vitro models. Our data nevertheless indicate that if IL-1β is released within the islets of the human pancreas, the cytokine will not necessarily cause the massive necrosis as is seen in cultured rat islets. To achieve this effect in human islets, local IL-1 levels will have to be chronically elevated and lead to toxic intercellular NO concentrations: this will probably require sufficient iNOS induction in

**TABLE 2**
Comparison of medium nitrite levels induced by IL-1β in rat islet cell preparations

<table>
<thead>
<tr>
<th>Islet cell preparation</th>
<th>Medium nitrites after 3 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/µl</td>
</tr>
<tr>
<td>Single β-cells (3 × 10⁶ cells/200 µl)</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>Aggregated β-cells (15 × 10⁶ cells/ml)</td>
<td>1.1 ± 0.2*</td>
</tr>
<tr>
<td>Islets (15 islets/ml)</td>
<td>1.8 ± 0.3†</td>
</tr>
</tbody>
</table>

Data are means ± SE from four independent experiments. Nitrite levels measured in medium collected after 3 days of culture were expressed per 10⁷ cells and/or per microgram of DNA at the start of culture. The statistical significance of difference with conditions of single β-cells was calculated by the two-sided paired t test: *P < 0.001, †P < 0.01. ND, not determined.

one or the other form of cell death depends on the presence of additional environmental factors.

Single rat β-cells were not killed by IL-1β when cultured at low plating densities (±90 cells/mm²) but underwent necrosis from day 3 on at high densities (±1,500 cells/mm²). Absence of IL-1–induced cytotoxicity in dispersed β-cell preparations (11,19,20, and present data) is thus not caused by an insensitivity to IL-1β, for example, as a result of the dissociation procedure or of the cellular attachment to dishes. It is not attributable to a lower NO production, as judged from the nitrite formation per 10⁷ β-cells, but rather is related to a higher distance between β-cells. Dilution of released NO may avoid toxic NO levels at neighboring cells. The role of NO-dependent cell necrosis indeed increases with plating density and is also high in cultures of reaggregated β-cells and of intact islets in which dilution is probably minimal. Our observations clarify the discrepancy that was previously noticed for the IL-1 effects in different in vitro models. They indicate that static cultures of compact islet cells can lead to toxic intercellular NO concentrations that cause necrosis. The question now is whether these NO levels can be reached in vivo, for example, when the flow in the islet interstitium is severely impaired.

The present in vitro experiments do not provide information on the effectors and mode of β-cell death in type 1 diabetes. This objective is difficult to achieve with in vitro models. Our data nevertheless indicate that if IL-1β is released within the islets of the human pancreas, the cytokine will not necessarily cause the massive necrosis as is seen in cultured rat islets. To achieve this effect in human islets, local IL-1 levels will have to be chronically elevated and lead to toxic intercellular NO concentrations: this will probably require sufficient iNOS induction in

**TABLE 3**
Cytotoxicity of NO donor GEA 3162 in rat and human islet cell preparations

<table>
<thead>
<tr>
<th>GEA 3162 (µmol/l)</th>
<th>Rat α-cells</th>
<th>Rat β-cells</th>
<th>Human islet cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 6</td>
<td>Day 1</td>
</tr>
<tr>
<td>25</td>
<td>10 ± 3%</td>
<td>&lt;5%</td>
<td>11 ± 3%*</td>
</tr>
<tr>
<td>50</td>
<td>80 ± 4%†</td>
<td>80 ± 2%†</td>
<td>11 ± 1%†</td>
</tr>
<tr>
<td>100</td>
<td>95 ± 1%†</td>
<td>94 ± 2%†</td>
<td>73 ± 5%†</td>
</tr>
</tbody>
</table>

Data are means ± SE from four independent experiments. The statistical significance of difference with control condition without GEA 3162 was calculated by the two-sided paired t test: *P < 0.05, †P < 0.001, ‡P < 0.01.
β-cells (41) or neighboring non-β-cells (21,22,40) as well as a reduced flow of the interstitial fluid. If these conditions are not met, the cytokine may cause apoptosis of β-cells if these are simultaneously and chronically exposed to other cytokines such as IFN-γ. Alternatively, IL-1β may influence the functional state of the β-cells by altering their phenotype (42) and, hence, their responses to physiological and pathophysiological agents.

ACKNOWLEDGMENTS

This work was supported by grants from Interuniversity Attraction Pole P4/21, the Belgian Fonds Wetenschappelijk Onderzoek—Vlaanderen (G.0376.97), and the Juvenile Diabetes Foundation (995004). A.H. was a research fellow (1993–1997) of the Belgian Fonds Wetenschappelijk Onderzoek—Vlaanderen; she is grateful to Miriam Marchal, head of the Pathology Department, and the other pathologists for taking over her clinical work during this period.

The authors thank their technical collaborators for preparing the rat and human β-cell preparations.

REFERENCES

38. Suarez-Pinzon WL, Styrnadka K, Schulz R, Rabinovitch A: Mechanisms of

