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Cytotoxic effects of cytokines on rat islets: evidence for involvement of free radicals and lipid peroxidation

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Summary. We have previously reported that oxygen free radical scavengers protect rat islet cells from damage by cytokines and we interpreted these findings as suggesting the involvement of oxygen free radicals but did not directly measure indices of free radical activity. In this study, we report on malondialdehyde, an end product of lipid peroxidation, in rat islets incubated with cytokines. The individual cytokines, interleukin 1 (1 U/ml), tumour necrosis factor (10^2 U/ml), and interferon gamma (10^2 U/ml) inhibited insulin release but did not increase islet malondialdehyde levels. Combination of these cytokines however, produced significant increases in islet malondialdehyde and this was accompanied by islet necrosis. Furthermore, an inhibitor of lipid peroxidation, U78518E, significantly decreased the cytokine-induced increase in islet malondialdehyde and protected islet Beta cells from destruction by the cytokine combination of interleukin 1, tumour necrosis factor and interferon gamma. These findings suggest that the cytotoxic action of cytokines on islet Beta cells may result from free radical production and lipid peroxidation in the islet cells.

Key words: islet Beta cells, oxygen free radicals, cytokines, interleukin 1, tumour necrosis factor, interferon gamma.

A variety of mononuclear cells of the immune system – T lymphocytes, macrophages, and natural killer cells, have been implicated in the islet Beta cell destruction associated with Type 1 (insulin-dependent) diabetes mellitus. It is not known, however, how these cells may mediate their islet Beta cell destructive effects. Effector mechanisms mediating islet Beta cell injury may involve direct contacts between immunologic cells (effectors) and islet Beta cells (targets), and/or may result from the release of soluble mediators by the effector cells.

Polypeptides (cytokines) produced by activated cells of the immune system are candidate molecules for the mediation of islet Beta cell autoimmune injury. Interleukin 1 (IL-1), a cytokine product of activated macrophages and natural killer cells, was the first cytokine reported to inhibit insulin release and/or to be cytotoxic to islets in vitro [1]. Subsequently, additional cytokine products of macrophages – tumour necrosis factor (TNF) and interleukin 6, and cytokines produced by T lymphocytes – interferon gamma (IFN-γ) and lymphotoxin, have been reported to impair insulin secretion and/or to destroy islet cells [2–5].

Mechanisms of cytokine-induced islet Beta cell injury are under active investigation [6, 7]. In a previous report, we obtained indirect evidence for involvement of oxygen free radicals in cytokine-mediated islet cell damage by using oxygen free radical scavengers to protect islet cells from damage by cytokines [8]. In the present study, we sought to obtain more direct evidence for oxygen free radical involvement in cytokine-mediated islet Beta cell destruction.

Materials and methods

Studies with whole islets

Pancreatic islets were isolated by collagenase digestion from adult Wistar-Furth rats (Charles River Canada, St. Constant, Quebec, Canada). The islets were incubated at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2, and maintained in free-floating culture in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY, USA) containing 10 % (volume/volume) heat-inactivated fetal calf serum (Gibco), 11 mmol/l glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 μg/ml amphotericin B, and 10 mmol/l HEPES buffer (complete medium). Experimental additions to the islets were made after 5 days of pre-incubation in complete medium.

Groups of 800–1200 islets were incubated at 37 °C for 20 to 80 h in 2.7 ml fresh complete medium with and without test substances in 35 × 10 mm Falcon tissue culture dishes (Becton Dickinson Laboratories, Lincoln Park, NJ, USA). Medium was collected for determination of insulin content by radioimmunoassay, using rat insulin standards and a kit (Pharmacia Fine Chemicals, Uppsala, Sweden). The islets were washed by centrifugation 3 times in Dulbecco's phos-

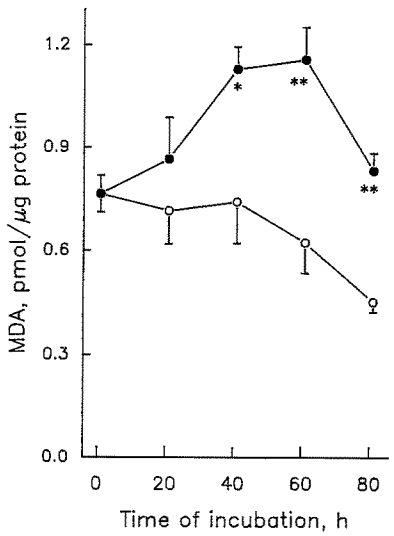

Fig. 1. Time-course effects of the cytokine combination of interleukin 1 (IL-1, 1 U/ml), tumour necrosis factor (TNF, 10^2 U/ml), and interferon gamma (IFN-γ, 10^2 U/ml) on malondialdehyde (MDA) levels in rat islets. Islets were incubated in medium without (○) and with (●) cytokines. Values are means ± SEM for five experiments. * $p < 0.05$, ** $p < 0.01$ vs control (cytokine-free) islet incubations

phate-buffered saline (Gibco). An aliquot was taken for assay of protein by the BCA (bicinchoninic acid) method (Pierce, Rockford, Ill., USA), and the malondialdehyde (MDA) content of the remaining islets was measured by the thiobarbituric acid reaction method [9]. In some experiments, islet MDA was measured by an ion-pairing high-performance liquid chromatography (HPLC) method [10].

Studies with islet cell monolayers

Islets were isolated, dissociated into single cells, and islet cell monolayer cultures were prepared in 96-well, half-area tissue culture plates (Costar, Cambridge, Mass., USA) by methods previously described [4].

For islet cell cytotoxicity studies, the monolayers were labelled by addition of 1 μCi ^{51}Cr sodium chromate (Dupont, Wilmington, Del., USA) to 170 μl complete medium per well. The plates were incubated at 37°C for 24 h, then washed 4 times, reincubated for 4 days in ^{51}Cr-free complete medium, and washed again 4 times. Test substances were added to the islet monolayers in complete medium (170 μl/well in quadruplicate) and the plates were incubated at 37°C for 4 days. An aliquot (100 μl) of supernatant medium was collected from each well and counted in a gamma counter. Percent specific cell lysis (cytotoxicity) was calculated as 100% × (test medium cpm − spontaneous cpm)/(total cpm − spontaneous cpm). Spontaneous release of ^{51}Cr was measured in wells incubated in medium alone and this was less than 15% of total ^{51}Cr release, measured by dissolving the cells in 4% Triton X-100 for 16 h.

To determine if cytotoxic or protective effects of agents on islet cells included Beta cells, test substances were added to unlabelled islet cell monolayers in complete medium and the plates were incubated at 37°C for 4 days. The monolayers were then washed 4 times and reincubated in fresh complete medium free of test substances for 3 days. This procedure was repeated, then the cells were washed in complete medium and extracted in acid-ethanol (75% ethanol, 1.5% 12 mmol/l HCl, and 23.5% H_2O) for assay of insulin content.

Cytokines and chemicals

Recombinant human IL-1β ($2–4 \times 10^7$ U/mg) was kindly provided by The Upjohn Co. (Kalamazoo, Mich., USA), recombinant human TNF-α (6×10^7 U/mg) and recombinant murine IFN-γ (8×10^6 U/mg) were kindly provided by Genentech (South San Francisco, Calif., USA). U78518E (kindly provided by The Upjohn Co.) is one of a novel class of antioxidants that are potent inhibitors of iron-dependent lipid peroxidation and protect against traumatic and ischaemic tissue damage [11]. U78518E was dissolved in methanol in a stock concentration of 10 mmol/l, then diluted in complete medium immediately before addition to the islet cells. Tert-butylhydroperoxide (t-BOOH) (Sigma, St. Louis, Mo., USA) was freshly prepared in distilled water and diluted in complete medium immediately before use.

Statistical analysis

Data are presented as means ± SEM, and statistical analyses were done with Student's two-tailed *t*-test for paired data.

Results

The time-course effects of the combination of IL-1 (1 U/ml), TNF (10^2 U/ml), and IFN-γ (10^2 U/ml) on islet levels of the lipid peroxidation end product, MDA, are shown in Figure 1. Since this cytokine combination is cytolytic to islet cells, causing a decrease in islet cell mass, MDA levels are expressed per μg islet protein. Increases in islet MDA were significant at 40 h ($p < 0.05$), 60 h ($p < 0.01$), and 80 h ($p < 0.01$) of incubation with the cytokines.

The effects of individual cytokines, and of the cytokine combination, were then examined in a 60 h incubation (Table 1). This shows that IL-1, TNF, and IFN-γ, as single agents, significantly inhibited insulin release, whereas IL-2 had no effect, and the combination of IL-1, TNF, and IFN-γ inhibited insulin release almost completely. Also, only the combination of IL-1, TNF, and IFN-γ was cytolytic to the islet cells, resulting in a significant decrease in islet protein. Similarly, only the combination of cytokines produced a significant increase in MDA levels in islets.

To determine whether the cytotoxic effects of cytokines might be related to increased peroxidation of islet lipids (increased MDA levels in islets), we tested an inhibitor of lipid peroxidation, U78518E. First, we determined that this substance could inhibit cytokine-induced increases in islet lipid peroxidation (Table 2). The combination of IL-1, TNF, and IFN-γ increased islet MDA from

Table 1. Effects of cytokines on insulin release, cell protein, and malondialdehyde (MDA) levels in rat islets

Cytokines (U/ml)	Insulin (nmol/dish)	Protein (μg/dish)	MDA (pmol/μg protein)
0	5340 ± 195	342 ± 32	0.64 ± 0.09
IL-1 1.0	1455 ± 150[b]	338 ± 57	0.59 ± 0.03
IL-2 1.0	5310 ± 345	313 ± 47	0.58 ± 0.09
TNF 10^2	3210 ± 75[a]	312 ± 55	0.85 ± 0.22
IFN-γ 10^2	2100 ± 345[a]	336 ± 41	0.72 ± 0.15
IL-1 1 + TNF 10^2 + IFN-γ 10^2	630 ± 90[b]	145 ± 32[a]	2.00 ± 0.30[a]

Incubation of 800–1000 rat islets was for 60 h in RPMI complete medium alone (0), and medium with cytokines, added individually and in combination as indicated. Values are means ± SEM for six experiments. [a] $p < 0.01$, [b] $p < 0.001$ vs corresponding value without cytokines. IL-1, interleukin 1; IL-2, interleukin 2; TNF, tumour necrosis factor; IFN-γ, interferon gamma

Table 2. Effects of a lipid peroxidation inhibitor U78518E on cytokine-induced increase in malondialdehyde (MDA) in rat islets

Cytokines (U/ml)	MDA (pmol/μg protein)		
	Without U78518E	With U78518E	p
0	0.26 ± 0.04	0.21 ± 0.02	NS
IL-1 1.0 + TNF 10^2 + IFN-γ 10^2	0.70 ± 0.21[a]	0.42 ± 0.13[a]	< 0.05

Incubation of 800–1200 rat islets was for 72 h in RPMI complete medium alone (0), and medium with the combination of cytokines shown, without and with U78518E (5 μmol/l). MDA levels in islets were measured by HPLC and mean values ± SEM are shown for four experiments. p values compare MDA in islets incubated without and with U78518E. [a] $p < 0.05$ vs corresponding value without cytokines. IL-1, interleukin 1; TNF, tumour necrosis factor; IFN-γ, interferon gamma

0.26 ± 0.04 pmol/μg protein to 0.70 ± 0.21 pmol/μg protein ($p < 0.05$), and U78518E reduced the cytokine-induced increase in MDA to 0.42 ± 0.13 pmol/μg protein ($p < 0.05$ vs cytokines alone).

We then tested U78518E for possible protection of islet cells against cytotoxic damage. This lipid peroxidation inhibitor (U78518E) provided similar dose-dependent protective effects against the standard oxidant, t-BOOH, and the cytokine combination of IL-1, TNF, and IFN-γ (Fig. 2). Thus, t-BOOH produced 36.6 ± 4.1 % lysis of islet cells and this was reduced to 14.4 ± 2.1 % (maximum protection) by 2.5 μmol/l U78518E. Similarly, the cytokines produced 41.2 ± 3.2 % lysis and this was reduced to 15.2 ± 2.0 % lysis (maximum protection) by 5 μmol/l U78518E.

To determine whether the protective effect of U78518E against cytokine-induced lysis of islet cells included protection of islet Beta cells, insulin content in the islet cell cultures was measured. Table 3 shows that the cytokines significantly decreased insulin content in the cultures (from 5475 ± 825 to 3000 ± 310 pmol insulin/well, $p < 0.05$). By contrast, insulin recovered in cells that had been incubated with cytokines plus U78518E (4425 ± 375 pmol insulin/well) was significantly greater than in cells that had been incubated with cytokines alone (3000 ± 310 pmol insulin/well, $p < 0.05$).

Discussion

In the present study, we have shown that a cytotoxic combination of cytokines (IL-1, TNF, and IFN-γ) increased levels of MDA, an end product of lipid peroxidation, in isolated rat pancreatic islets. Furthermore, an inhibitor of lipid peroxidation (U78518E) reduced the cytokine-induced increase in MDA levels in islets, and protected islet cells from the cytotoxic effects of both the cytokine combination and a standard oxidant, t-BOOH. The protective effect of the lipid peroxidation inhibitor against the cytodestructive effects of the cytokines included protection of islet Beta cells. These data suggest that cytokine-induced cytotoxicity to islet Beta cells is causally related to lipid peroxidation events in the islet cells. Since lipid peroxidation is a major mechanism of oxygen free radical toxicity to cellular organelles and membrane-bound enzymes [12], we interpret the present data to implicate oxygen free radical-induced lipid peroxidation as a mechanism of cytokine-induced cytotoxicity to islet Beta cells.

Whereas the present study implicates oxygen free radicals in the cytotoxic (lytic) effects of cytokines on islet Beta cells, oxygen free radicals do not appear to be involved in the inhibitory effects of cytokines on insulin secretion unaccompanied by cell destruction. Thus, we found that all three individual cytokines – IL-1, TNF, and IFN-γ inhibited insulin release significantly, as single agents; however islet protein content was not reduced and there was no increase in islet lipid peroxidation. These findings are in agreement with those of another study which found no protective effects of oxygen free radical scavengers on IL-1-induced inhibition of insulin release [13]. Therefore, inhibition of insulin release by IL-1, or by other individual cytokines (TNF, IFN-γ) does not appear to involve oxygen free radical-mediated mechanisms. By contrast, the destructive effects to islets of a combination of cytokines does appear to involve oxygen free radical production, as we have previously suggested by using oxygen free radical scavengers [8], and as supported by this study in which we have detected increased lipid peroxidation in islets incubated with a cytodestructive combination of cytokines.

The cytokines IL-1, TNF, and IFN-γ have been reported to stimulate oxygen free radical production during the respiratory burst in a variety of cell types, such as macrophages [14], and polymorphonuclear leucocytes [15] in association with host defence against microorganisms and tumours. Stimulation of oxygen free radical production in pancreatic islet cells, however, might be expected to have

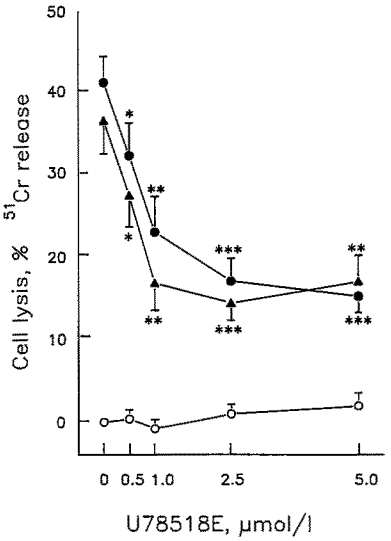

Fig. 2. Dose-dependent protective effects of the lipid peroxidation inhibitor U78518E on rat islet cell lysis produced by the cytokine combination (●) of interleukin 1 (IL-1, 1 U/ml), tumour necrosis factor (TNF, 10^2 U/ml), and interferon gamma (IFN-γ, 10^2 U/ml), and by the oxidant, tert-butylhydroperoxide (t-BOOH, 0.1 mmol/l, ▲) during 4-day incubations of islet cell monolayers. Cell lysis in incubations with U78518E alone are shown as (○). Values are means ± SEM for five experiments. * $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$ vs no U78518E inhibitor

Table 3. Effects of a lipid peroxidation inhibitor U78518E on cytokine-induced decrease in insulin content in rat islet cells

Cytokines (U/ml)	Insulin content (pmol/well)		
	Without U78518E	With U78518E	*p*
0	5475 ± 825	5040 ± 540	NS
IL-1 1.0 + TNF 10^2 + IFN-γ 10^2	3000 ± 310[a]	4425 ± 375	< 0.05

Rat islet cell monolayer cultures were incubated for 4 days in RPMI complete medium alone (0), and medium with the combination of cytokines shown, without and with U78518E (5 μmol/l). The monolayers were then washed 4 times and re-incubated in fresh medium free of cytokines and U78518E for 3 days. This procedure was repeated, then the cells were washed in complete medium and extracted in acid-ethanol for assay of insulin content. Mean values ± SEM are shown for four experiments. *p* values compare insulin in cells incubated without and with U78518E. [a] $p < 0.05$ vs corresponding value without cytokines. IL-1, interleukin 1; TNF, tumour necrosis factor; IFN-γ, interferon gamma

lethal consequences to islet cells since these cells possess very low free radical scavenging enzyme activities [16, 17], and are extremely vulnerable to free radical-induced injury [17].

The mechanisms by which cytokines might stimulate oxygen free radical production in pancreatic islets are unknown. Cytokine-induced oxygen free radicals are primarily products of arachidonic acid metabolism in many cell types, and we have observed that inhibitors of arachidonic acid metabolism can protect islet cells from the cytotoxic effects of TNF and IFN-γ [18]. These data implicate arachidonate metabolites (eicosanoids) in cytokine-induced islet cell injury. Furthermore, increased metabolism of arachidonic acid can give rise to free radical intermediates, and free radicals, in turn, can regulate arachidonic acid metabolism and amplify eicosanoid synthesis [19].

Whereas lipid peroxidation is commonly regarded as the consequence of oxygen free radical-mediated injury, nitrogen-centred free radicals may also contribute to lipid peroxidation [20]. The possibility that nitrogen free radicals may be involved in cytokine actions on islet cells is supported by a recent report that IL-1 and TNF-induced inhibition of insulin release from islets in vitro involved the nitrogen free radical, nitric oxide [21]. In addition, activated macrophages have been reported to kill islet cells in vitro via nitric oxide generation [22].

Evidence exists to suggest involvement of oxygen free radicals in immune-mediated damage to islet Beta cells in vivo. Thus, desferrioxamine, an inhibitor of hydroxyl radical formation, and nicotinamide, a poly (ADP-ribose) synthetase inhibitor and a weak free radical scavenger, protected islet cells from immune destruction in allograft rejection and in low-dose STZ-induced insulitis [23]. Also, treatment of spontaneously diabetic non-obese mice with nicotinamide and desferrioxamine [24], or with superoxide dismutase [25], protected islet grafts from immune damage and consequent recurrence of diabetes. In addition, we have found recently that the lipid peroxidation inhibitor (U78518E) which protected rat islets from the cytotoxic effects of cytokines in the present study in vitro, also prevented the development of diabetes in non-obese mice in vivo (unpublished observation). We are presently investigating whether the protective effects of U78518E against diabetes development in vivo might be through interference with cytokine-induced oxygen free radical production and lipid peroxidation events in islet cells, as suggested by the present study with U78518E in vitro.

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