

# Effects of prolonged exposure to pancreatic glucagon on the function, antigenicity and survival of isolated human islets

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## Abstract

**Background** Certain clinical conditions are associated with inappropriately high levels of circulating glucagon. To date, little information is available about the direct effects of prolonged exposure of human islet cells to pancreatic glucagon. In the present study we evaluated the function, antigenicity and survival of human islets exposed for 24 h to human pancreatic glucagon.

**Methods** We prepared human islets of Langerhans by collagenase digestion and density-gradient purification, incubated them for 24 h with 44 or 430 pmol/l pancreatic glucagon at physiological (5.5 mmol/l) glucose level, and evaluated their insulin release function, which was then compared with that obtained from islets kept at high (11.1 mmol/l) glucose concentration. In addition, aliquots of the islets were evaluated to assess their chemotactic properties towards human monocyte-macrophage cells, and their potency to induce cytokine release from human lymphocytes. Finally, survival of the islet cells cultured under varying conditions was evaluated, and an assessment was performed of mRNA expression of Bcl-2 and Bax proteins.

**Results** The insulin secretion results demonstrated that, compared to the control islets, the islets previously exposed to either 44 or 430 pmol/l glucagon exhibited changes in insulin release in response to glucose, consisting of augmented secretion at low glucose challenge, and no further significant increase at high glucose stimulation, similar to the effects observed with islets pre-cultured with high glucose. These effects were reversible, as documented by the recovery of normal islet sensitivity to glucose after an additional 24-h culture in medium lacking glucagon. Compared to control islets, the culture medium from islets pre-cultured with high glucagon or high glucose showed an increased chemotactic potency towards human monocyte-macrophage cells. In addition, human lymphocytes released a greater amount of tumour necrosis factor alpha when co-cultured with the islets pre-exposed to high glucagon or high glucose, whereas no significant difference was observed (in comparison with control islets) as regards the release of gamma-interferon, interleukin-2 and interleukin-10. The TUNEL technique and RT-PCR showed, respectively, no major difference in cell survival and expression of mRNA encoding for Bcl-2 and Bax protein between control islets and islets kept for 24 h in the presence of high glucagon or high glucose.

**Conclusions** Our results show that *in vitro* exposure of human islets to pancreatic glucagon for 24 h causes changes in the function and antigenicity of isolated human islets that are similar to the changes observed after pre-

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culture with increased glucose levels. Under our experimental conditions, these changes were not accompanied by any evidence of cytotoxicity. Copyright © 2000 John Wiley & Sons, Ltd.

**Keywords** glucagon; human islets; insulin release; chemotaxis; cytokines; Bcl-2

## Introduction

Insulin release is a complex phenomenon, finely regulated by the interplay between nutrients, neurotransmitters and hormones [1,2]. Much information is currently available about the effects on insulin secretion of acute exposure to several physiologic or pharmacologic compounds [1,2]. However, little is known about the action of prolonged exposure of islet cells to insulin secretagogues, with the notable exception of glucose [3–5]. Indeed, in the past few years it has been clearly demonstrated that pre-exposure of pancreatic islets to high glucose concentrations *in vitro* impairs beta cell function, providing experimental evidence in support of the concept that chronic hyperglycemia can further damage islet insulin secretion [3–5]. In addition, it has been reported that exposure to high glucose increases islet antigenicity [6,7], suggesting a link between the functional status and immunological properties of pancreatic islets.

Glucagon is the major product of pancreatic islet alpha cells [8]. Its main physiologic effect is the regulation of the rates of glucose production due to both glycogenolysis and gluconeogenesis. In this respect, insulin antagonizes the action of glucagon, such that the relative concentrations of the two hormones determine the eventual effect on insulin production. Insulin and glucagon may also affect the respective secretion rates: insulin can reduce glucagon release [8], and glucagon can increase insulin secretion acutely [9].

To date, little information is available about the direct effects of prolonged exposure of islet cells to glucagon on insulin release. A previous study demonstrated that 4 h incubation with glucagon did not affect the function of human islets [10], however data on the possible effects of more prolonged exposure is lacking. Since certain clinical conditions, such as Type 2 diabetes [11,12], stress hyperglycemia [13] and glucagonoma [14] are associated with inappropriately high levels of circulating glucagon and altered insulin secretion, we considered it of interest to evaluate whether prolonged exposure of human islets to glucagon might directly affect the insulin secretion function of isolated human pancreatic islet cells. In addition, we performed experiments to assess whether the possible functional changes of the glucagon-exposed cells were paralleled by changes in the antigenic properties of the islets, or cytotoxicity. The results were compared with those obtained from islets pre-cultured in the presence of high concentrations of glucose.

## Materials and methods

### Islet preparation

The procedures for islet preparation were based on collagenase digestion and density-gradient purification as previously described [15,16]. In the present study we used islets from the pancreases of five human cadaver donors, three males and two females, aged 18–48 years. Three additional pancreases (two males and one female, aged 25–39 years) were used for the TUNEL technique experiments (see later). All the protocols were approved by our local Ethics Committee.

### Insulin secretion studies

We tested the effect of pancreatic glucagon, pancreatic GLP-1 (1–37), and high glucose concentrations on the function of isolated human islets. The islet cells were kept for 24 h in either M199 culture medium, or in M199 medium containing either 44 pmol/l pancreatic glucagon, 430 pmol/l pancreatic glucagon, 10 nmol/l pancreatic GLP-1 (1–37), or 11.1 mmol/l glucose. With the exception of the last experimental condition, the glucose concentration in the incubation medium was 5.5 mmol/l. The proteinase inhibitor Trasylol was also added at a concentration of 500 U/ml to prevent possible incubation damage of glucagon [17] and GLP-1 (1–37). The effect of Trasylol alone on islet function was tested, and found to have a negligible effect (data not shown). All the reagents described were purchased from Sigma Chemicals, St Louis, MO, USA. At the end of the culture periods, the islets were evaluated for their insulin response to varying glucose concentrations, according to standard procedures employed in our laboratory [15,16]. Following a 30-min pre-incubation period at 3.3 mmol/l glucose, batches of five to ten islets of comparable size (100–200 µm diameter) were tested at 37°C for 45 min in Krebs-Ringer bicarbonate solution, 0.5% albumin, pH 7.4, containing either 3.3 or 16.7 mmol/l glucose. At the end of the glucose challenge, the number of islets was confirmed by dithizone staining.

We also tested whether the possible effects of glucagon on islet function were reversible. To do this, at the end of the 24-h incubation period the islets were washed, cultured for an additional 24 h in control M199 medium, and challenged once again with low and high glucose concentrations.

Insulin levels in the incubation media were measured using a commercially available insulin IRMA kit (Medgenix, Brussels, Belgium) and the levels expressed as picomoles/islet/45 min.

### Islet antigenicity studies

Human lymphomononuclear cells (LMC) and activated monocyte-macrophage cells (MM) were prepared from healthy volunteers as described previously [18,19]. LMC were isolated by differential density-gradient centrifuga-

gation of peripheral venous blood from healthy volunteers. Aliquots of these cells were kept at 37°C on plastic in M199 culture medium for 60 min, so as to allow the cells of the monocyte-macrophage series to adhere. The non-adherent lymphocytes were removed by washing with fresh medium, and their ability to release lymphokines was assessed as described below. The cells adhering to the plastic were maintained at 37°C for 48 h to permit activation to occur. The activated MM were finally detached from the culture flasks by incubation for 15 min at 4°C. Lymphocytes and MM viability, as evaluated by the trypan blue exclusion technique at the end of the preparation procedures, was >90%.

A modified chemotaxis Boyden chamber was used as described by Kessler *et al.* [19] to evaluate the chemotaxis of activated human MM towards aliquots of the culture media in which the islets had been kept for 24 h. In each experiment we evaluated how many MM moved to the membrane of the chamber in the presence of either the cytotoxin formyl-metionin-leucin peptide (fMLP, diluted 1:10<sup>8</sup> in M199), control M199 medium, or the supernatant from the islets previously kept for 1 day in 5.5 mmol/l glucose without glucagon, 5.5 mmol/l glucose plus 430 pmol/l glucagon, or 11.1 mmol/l glucose. Medium containing 430 pmol/l glucagon or 11.1 mmol/l glucose, without islets, was also tested. The results were computed by counting the number of migrated MM on a light microscope, and expressed as the ratio of migrated cells at a given condition over the number of cells migrated in the presence of control M199 medium (chemotactic index) [19]. In each experiment ten fields were counted.

The methods we used to evaluate the release of cytokines from human lymphocytes have been described in detail previously [20]. We co-cultured 1 × 10<sup>6</sup> lymphocytes for 3 days with approximately 300 hand-picked human islets previously maintained for 1 day in control M199 medium, or in medium containing 430 pmol/l glucagon or 11.1 mmol/l glucose. At the end of the 3-day culture period, tumour necrosis factor alpha (TNF), gamma-interferon (IFN), interleukin-2 (IL-2), and interleukin-10 (IL-10) concentrations were measured by sandwich enzyme-linked immunoadsorbent assay (Medgenix Diagnostics, Fleurus, Belgium).

## Islet survival studies

Islet cell survival was assessed by the TUNEL technique. The TUNEL DNA fragmentation kit (Boehringer Mannheim, Germany) was used in accordance with the procedures outlined by the manufacturer.

The expression of mRNA encoding for Bcl-2 and Bax proteins, two proteins involved in determining the fate of cells (survival or death by apoptosis) was evaluated by RT-PCR. Total RNA was extracted from purified human pancreatic islets with Trizol (Gibco-BRL, Grand Island, NY, USA) in accordance with the manufacturer's instructions. First-strand cDNA synthesis was performed using 2 µg of each RNA sample primed with random

examers with 200 U of Superscript II (Gibco-BRL); 200 ng aliquots of cDNA were subsequently amplified in 100 µl reaction volume containing 20 pmol of upstream- and downstream-specific primers, 2.5 U of Taq DNA polymerase (Gibco-BRL), 200 µM of each deoxynucleoside triphosphate and 1.5 mM MgCl<sub>2</sub>. The human Bcl2 primer pair (5'-ACA ACA TCG CCC TGT GGA TGA C-3' and 5'-ATA GCT GAT TCG ACG TTT TGC C-3') and human Bax primer pair (5'-GGC CCA CCA GCT CTG AGC AGA-3' and 5'-GCC ACG TGG GCG TCC CAA AGT-3') generated a 408 bp and a 477 bp product, respectively. Expression of beta-actin as RNA control was analyzed employing the following primers generating a 354 bp product (5'-ACC AAC TGG GAG GAG ATG GAG-3' and 5'-CGT GAG GAT CTT CAT GAG GTA AGT C-3'). All PCR products were electrophoresed on 1.2% Separide agarose gel and bands visualized by ethidium bromide staining.

## Data analysis

Results are expressed as mean ± SEM. Statistical analysis of the data was performed by ANOVA, the Bonferroni test, or the two-tailed unpaired Student's *t*-test, as appropriate (see Results).

## Results

Table 1 shows insulin release results in response to 3.3 and 16.7 mmol/l glucose from human islets exposed for 24 h to either glucagon, GLP-1 (1–37), or high glucose. Compared to the control islets, the islets previously exposed to either 44 or 430 pmol/l glucagon or 11.1 mmol/l glucose showed significantly higher insulin release in response to 3.3 mmol/l glucose, whereas the secretion at 16.7 mmol/l glucose did not differ significantly between the various experimental groups. On the whole, this caused no significant change in insulin release at the high vs the low glucose concentration from the islets previously exposed to glucagon or high glucose. Conversely, the islets pre-cultured with pancreatic GLP-1 (1–37) showed insulin release characteristics similar to those of the control islets.

When the islets that had been exposed to glucagon for 1 day were placed in the glucagon-free control M199 culture medium for an additional 24 h, they regained their sensitivity to glucose. In this experiment, insulin release at 3.3 mmol/l glucose was 17.7 ± 2.6 and 17.2 ± 1.3 pmol/islet from the islets pre-exposed to 44 or 430 pmol/l glucagon, respectively, and then washed, and the corresponding values at 16.7 mmol/l glucose were 46.2 ± 6.3 and 42.4 ± 3.8 pmol/islet (*p* < 0.01 vs 3.3 mmol/l glucose).

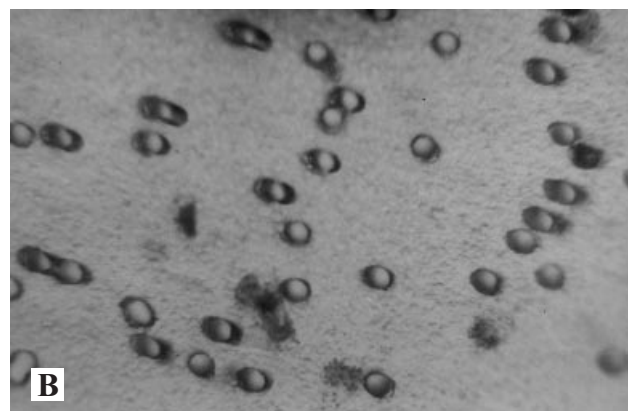
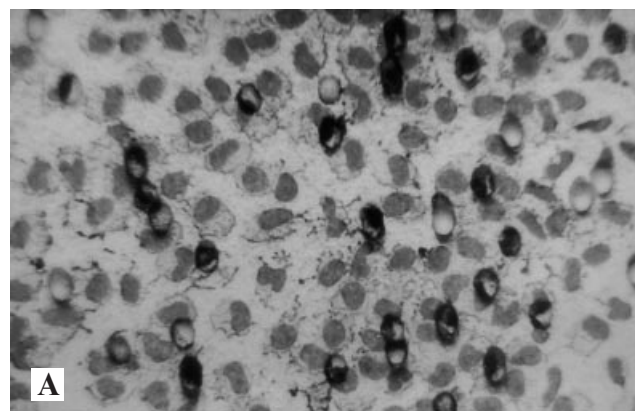
Figure 1 shows an example of the appearance of activated MM after they had moved to the membrane in the Boyden chamber in response to fMLP or control medium. When the MM were challenged with the medium from control islets, the supernatant from islets

**Table 1. Insulin release (picomoles/islet) in response to glucose from isolated human islets pre-incubated for 24 h with pancreatic glucagon, pancreatic GLP-1 (1–37), or high concentrations of glucose<sup>a</sup>**

Pre-incubation	Insulin release at 3.3 mmol/l glucose	Insulin release at 16.7 mmol/l glucose	P value (Student's <i>t</i> -test)
Control medium	14.38 ± 0.34 (20)	39.55 ± 6.81 (16)	<0.01
Glucagon (44 pmol/l)	29.22 ± 6.60 (10)	43.57 ± 8.05 (11)	NS
Glucagon (430 pmol/l)	31.67 ± 11.77 (10)	45.67 ± 12.12 (8)	NS
GLP-1 (10 nmol/l)	17.78 ± 1.79 (19)	35.91 ± 2.02 (16)	<0.01
Glucose (11.1 mmol/l)	26.0 ± 3.1 (16)	36.1 ± 7.0 (16)	NS
P value (ANOVA)	<0.05	NS	—

<sup>a</sup>Number of replicates is given in parentheses.

NS, not significant.

**Figure 1. Monocyte-macrophages at the membrane level of a modified Boyden chamber in response to (A) fMLP or (B) control medium**

cultured for 24 h with high glucagon or high glucose concentration, the respective chemotactic indices were  $1.3 \pm 0.1$ ,  $1.9 \pm 0.2$  and  $1.8 \pm 0.1$  ( $p < 0.05$  by ANOVA). Medium without islets that contained added high glucagon or high glucose did not cause any significant increase in the chemotactic index ( $1.2 \pm 0.1$  and  $1.3 \pm 0.1$ , respectively).

Table 2 shows the release of cytokines from human lymphocytes (LC) cultured for 3 days with control islets or islets pre-exposed to high glucagon or high glucose. As expected [20] the release of the tested cytokines was higher from LC tested with the islets than from LC alone (data not shown). The amount of TNF released by human LC co-cultured for 3 days with the islets previously maintained in the presence of high glucagon or high glucose was similarly and significantly higher than the amount secreted with control islets. The production of the other measured cytokines did not differ, regardless of the experimental conditions. Medium containing high

glucagon or high glucose without islets did not affect the release of the cytokines, compared to plain medium (data not shown).

The TUNEL technique demonstrated that the number of dead cells was similar in the control islets ( $14 \pm 4\%$ ) and in islets that had been cultured for 24 h in the presence of high glucagon ( $16 \pm 3\%$ ) or high glucose ( $13 \pm 4\%$ ) concentration. Accordingly, no major change in the expression of mRNA encoding for Bcl-2 and Bax was found after culture with either high glucose or high glucagon concentrations (Figure 2).

## Discussion

The results of our study show that pre-exposure of isolated human islets to pancreatic glucagon for 24-h altered islet insulin secretory function. This effect was similar to that observed with high glucose, it was not observed with pancreatic GLP-1 (1–37), and it was reversible. These functional changes were accompanied by changes in islet antigenicity, without evidence of cytotoxicity.

It is known that glucagon binds to specific receptors on beta cells [9] and promotes insulin secretion, possibly by increasing intracellular cyclic-AMP [21]. Since a prolonged enhancement of insulin release may alter islet cell function (a phenomenon that has been consistently observed – and confirmed in the present study – when the islets are exposed to high glucose levels) [3–5], one

**Table 2. Cytokine release from human lymphocytes co-cultured for 3 days with human islets previously incubated under varying culture conditions<sup>a</sup>**

Culture conditions	TNF alpha (pg/ml)	IFN gamma (IU/ml)	IL-2 (IU/ml)	IL-10 (pg/ml)
Control medium	74 ± 7	1.12 ± 0.1	1.35 ± 0.06	9.3 ± 1.0
Glucagon (430 pmol/l)	216 ± 6*	1.13 ± 0.09	1.52 ± 0.1	13.3 ± 1.2
Glucose (11.1 mmol/l)	208 ± 11*	1.03 ± 0.1	1.60 ± 0.2	12.8 ± 1.5

<sup>a</sup>Mean ± SEM of five replicates.\* $p < 0.05$  vs control medium.

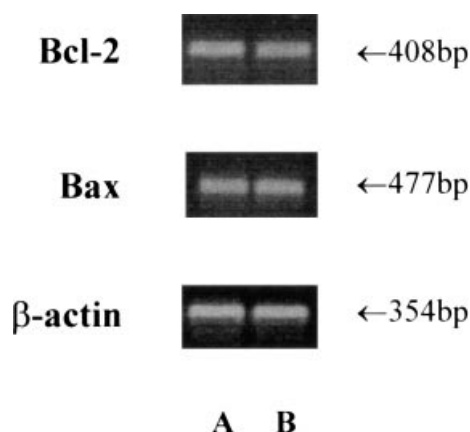


Figure 2. Bcl-2 and Bax mRNA expression in (A) control islets and (B) islets exposed to glucagon

can speculate that the altered islet function after pre-exposure to glucagon could be due, at least in part, to the prolonged stimulatory effect of the hormone on the beta cells. Indeed, when we pre-cultured the human islets with pancreatic GLP-1 (1–37) at a concentration (10 nmol/l) known to have no insulinotropic effect [22], insulin release in response to glucose was well preserved.

The altered insulin release due to glucagon was mainly observed in response to 3.3 mmol/l glucose. At this glucose concentration, insulin secretion increased, an effect that again is similar to that found after exposure of the islets to high glucose concentrations [3–5, the present study]. A possible explanation in the case of exposure to high glucose is an enhanced activity of the low- $K_m$  glucose-phosphorylating activity, due to hexokinase [23,24]. The molecular mechanisms involved in the case of glucagon remain to be elucidated.

In the present study, the effect of glucagon was also found at 'physiological' concentration (44 pmol/l). Glucagon release *in vivo* is pulsatile [25], whereas, for obvious reasons, in our *in vitro* study the islets were exposed to non-oscillating glucagon levels, and this may have amplified the biological action of the hormone on the islet cells.

The effect of glucagon on islet insulin release in response to glucose observed in the present study was reversible after the alpha cell hormone was removed. From our experiments it is not possible to determine whether a longer exposure period could lead to a more pronounced and irreversible change. This issue will require further investigation.

It has been shown previously that islets exposed to high glucose show increased antigenicity [6,7]. This suggests that an enhanced function might exacerbate the autoimmune attack in the early phases of Type 1 diabetes, and also alter the viability and survival of islet grafts. The present study reports that pre-culture with high glucose or high glucagon increases the chemotactic potency of the supernatant from human islets, and their ability to elicit the release of TNF from lymphocytes. The effect on MM might be due to the action of insulin itself, even though this remains questionable [26]. In any case, a functional

hyperactivity of the islets seems to be associated with an increase in the pro-inflammatory properties of the islets themselves, reinforcing the importance of strategies aiming to bring about islet rest in certain conditions, such as, for instance, pancreas or islet transplantation.

From a clinical standpoint, our *in vitro* findings permit some speculation. For example, high glucagon levels are often found in diabetic patients [11,12], and this might have consequences on the residual islet cell function. In addition, although only from a speculative point of view, the markedly reduced success rate of islet transplant therapy in patients with Type 1 diabetes, compared to islet autografts and islet allografts in patients rendered apantretic by upper-abdominal exenteration [27–30], might be due, at least in part, also to the glucagon released from the native pancreas (that remains *in situ* in patients with Type 1 diabetes, whereas it has been removed in the other groups). The alpha cell hormone reaching the intrahepatic microenvironment (at concentrations that are conceivably higher than in the systemic circulation) where the islets are lodged might therefore disturb intrahepatic islet engraftment and function. Indirect support for this hypothesis comes from the well-known evidence that intrahepatic islets tend to fail even in the case of islet isografts [31], a phenomenon that is not observed, for example, when the islets are implanted under the kidney capsule [32]. In this regard, since under our experimental conditions no major effect on islet survival or the expression of mRNA encoding for the anti-apoptotic and pro-apoptotic proteins Bcl-2 and Bax [33,34] was observed by incubation with increased glucose or glucagon levels, it seems likely that the possible deleterious effect of glucose and glucagon on islet cell survival are not direct, but possibly mediated by increased islet antigenicity, as discussed above.

In conclusion, the results of our study show that prolonged *in vitro* exposure of human islets to glucagon causes changes in the function and antigenicity of isolated human islets that are similar to those observed after pre-culture with increased glucose levels, and raise the possibility that clinical conditions exposing the islets to increased glucagon levels may contribute to causing islet damage.

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## References

1. Cook DL, Taborsky GJ. Beta cell function and insulin secretion. In *Ellenberg & Rifkin's Diabetes Mellitus*, Porte D Jr, Sherwin RS (eds). Appleton & Lange: Stamford, Connecticut, 1997; 49–74.
2. Malaisse WJ. Insulin biosynthesis and secretion *in vitro*. In *International Textbook of Diabetes Mellitus*, Alberti KGMM, DeFronzo RA, Keen H, Zimmet P (eds). John Wiley & Sons: Chichester, 1996; 261–284.

3. Robertson RP, Olson LK, Zhang HJ. Differentiating glucose toxicity from glucose desensitization: a new message from the insulin gene. *Diabetes* 1994; **43**: 1085–1089.
4. Eizirik DL, Korbitt GS, Hellerstrom C. Prolonged exposure of human pancreatic islets to high glucose concentrations *in vitro* impairs the B-cell function. *J Clin Invest* 1992; **90**: 1263–1268.
5. Davalli AM, Ricordi C, Socci C, *et al.* Abnormal sensitivity to glucose of human islets cultured in a high glucose medium: partial reversibility after an additional culture in a normal glucose medium. *J Clin Endocrinol Metab* 1991; **72**: 202–208.
6. Bjork E, Kampe O, Karlsson FA, *et al.* Glucose regulation of the autoantigen GAD65 in human pancreatic islets. *J Clin Endocrinol Metab* 1992; **75**: 1574–1576.
7. Ekblond A, Schou M, Buschard K. Mononuclear cytotoxicity and proliferation towards glucose stimulated rodent pancreatic islet cells. *Autoimmunity* 1997; **25**: 97–108.
8. Lefebvre PJ. Glucagon and its family revisited. *Diabetes Care* 1995; **18**: 715–730.
9. Moens K, Flamez D, Van Schravendijk C, Ling Z, Pipeleers D, Schuit F. Dual glucagon recognition by pancreatic beta-cells via glucagon and glucagon-like peptide 1 receptors. *Diabetes* 1998; **47**: 66–72.
10. Bertuzzi F, Berra C, Socci C, Davalli AM, Pozza G, Pontiroli AE. Insulin and glucagon release of human islets *in vitro*: effects of chronic exposure to glucagon. *J Endocrinol* 1997; **152**: 239–243.
11. Unger RH, Orci L. Glucagon and the alpha cell. Physiology and pathophysiology. *N Engl J Med* 1981; **304**: 1518–1524.
12. Muller WA, Faloon GR, Unger RH. The effect of experimental insulin deficiency on glucagon secretion. *J Clin Invest* 1971; **50**: 1992–1999.
13. Park CR, Exton JH. Glucagon and the metabolism of glucose. In *Glucagon: Molecular Physiology, Clinical and Therapeutic Implications*, Lefebvre PJ, Unger RH (eds). Pergamon Press: Oxford, 1972; 77–108.
14. Polak JM, Bloom SR. Glucagon-producing tumors and the glucagonoma syndrome. In *Endocrine Pathology of the Gut and Pancreas*, Dayal Y (ed.). CRC Press: Boca Raton, 1991; 227–240.
15. Marchetti P, Dotta F, Ling Z, *et al.* The function of pancreatic islets isolated from Type 1 diabetic patients. *Diabetes Care* 2000; **23**: 701–703.
16. Pupilli C, Giannini S, Marchetti P, *et al.* Autoantibodies to CD38 (ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase) in Caucasian patients with diabetes: effects on insulin release from human islets. *Diabetes* 1999; **48**: 2309–2315.
17. Eisentraut AM, Whissen N, Unger R. Incubation damage in the radioimmunoassay for human plasma glucagon and its prevention with Trasylol. *Am J Med Sci* 1968; **33**: 137–142.
18. Boyum A. Separation of leucocytes from blood and bone marrow. *Scand J Clin Lab Invest* 1968; **21**: 77–89.
19. Kessler L, Jesser C, Lombard Y, *et al.* Cytotoxicity of peritoneal murine macrophages against encapsulated pancreatic rat islets: *in vivo* and *in vitro* studies. *J Leukoc Biol* 1996; **60**: 729–736.
20. Marselli L, Marchetti P, Tellini C, *et al.* Lymphokine release from human lymphomononuclear cells after co-culture with isolated pancreatic islets: effects of islets species, long-term culture, and monocyte-macrophage cell removal. *Cytokine* 2000; **12**: 503–505.
21. Rodbell M. The actions of glucagon at its receptor: regulation of adenylate cyclase. In *Glucagon I: Handbook of Experimental Pharmacology* 66/1, Lefebvre PJ (ed.). Springer-Verlag: Heidelberg, 1983; 263–290.
22. Kawai K, Suzuki S, Ohashi S, *et al.* Comparison of the effects of glucagon-like peptide-1 (1–37) and (7–37) and glucagon on islet hormone release from isolated perfused canine and rat pancreases. *Endocrinology* 1989; **124**: 1768–1773.
23. Hosokawa H, Hosokawa YA, Leahy JL. Upregulated hexokinase activity in isolated islets from diabetic 90% pancreatectomized rats. *Diabetes* 1995; **44**: 1328–1333.
24. Purrello F, Rabuazzo AM, Anello M, Patanè G. Effects of prolonged glucose stimulation on pancreatic beta cells: from increased sensitivity to desensitization. *Acta Diabetol* 1996; **33**: 253–256.
25. Paolisso G, Scheen AJ, Albert A, Lefebvre PJ. Effects of pulsatile delivery of insulin and glucagon in humans. *Am J Physiol* 1989; **257**: E686–E696.
26. Karsten V, Lencioni C, Tritscher S, *et al.* Chemotactic activity of culture supernatants of free and encapsulated pancreatic rat islets towards peritoneal macrophages. *Horm Metab Res* 1999; **31**: 448–454.
27. Scharp DW, Lacy PE, Santiago JV, *et al.* Results of our first nine intraportal islet allografts in Type 1, insulin-dependent diabetic patients. *Transplantation* 1991; **51**: 76–85.
28. Warnock GL, Kneteman NM, Ryan E, Seelis REA, Rabinovitch A, Rajotte RV. Normoglycaemia after transplantation of freshly and cryopreserved pancreatic islets in Type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 1991; **34**: 55–58.
29. Ricordi C, Tzakis A, Carroll P, *et al.* Human islet transplantation in 18 diabetic patients. *Transplant Proc* 1992; **24**: 961–962.
30. Socci C, Falqui L, Davalli AM, *et al.* Fresh human islet transplantation to replace pancreatic endocrine function in type I diabetic patients. *Acta Diabetol* 1991; **28**: 151–157.
31. Mellgren A, Schnell AH, Petersson B, Andersson A. The renal subcapsular site offers better growth conditions for transplanted mouse pancreatic islet cells than the liver or the spleen. *Diabetologia* 1986; **29**: 670–672.
32. Hiller WFA, Klempnauer J, Luck R, Steiniger B. Progressive deterioration of endocrine function after intraportal but not kidney subcapsular rat islet transplantation. *Diabetes* 1991; **40**: 134–140.
33. Di Mario U, Dotta F. Beta cell autoimmunity 1998. *Diabetes Metab Rev* 1998; **14**: 1–2.
34. Benoist C, Mathis D. Cell death mediators in autoimmune diabetes. No shortage of suspects. *Cell* 1997; **89**: 1–3.