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Danny Pipeleers Minkowski Award, 1986, Rome

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Physiologic relevance of heterogeneity in the pancreatic beta-cell population

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Summary In vitro studies on purified rat beta cells have indicated a functional diversity among insulin-containing cells. Intercellular differences were found in the rates of glucose-induced insulin synthesis and release. They are attributed to differences in cellular thresholds for glucose utilization and oxidation, as can be caused by varying activities in rate limiting steps such as glucokinase-dependent phosphorylation. The percent of functionally active beta cells increases dose-dependently with the glucose concentration, making cellular heterogeneity and its regulation by glucose major determinants for the dose-response curves of the total beta-cell population. Beta cells which are already responsive to low glucose concentrations are characterized by a higher content in pale immature granules; their activated biosynthetic and secretory activity accounts for preferential release of newly-formed hormone by the total beta-cell population. At any glucose level, the amplitude of insulin release depends on the percent glucose-activated cells and their cyclic AMP content, an integrator of (neuro)hormonal influences. The in vitro described heterogeneity in beta-cell functions may bear physiological relevance as several of its characteristics are also detectable in intact pancreatic tissue; furthermore, in vitro signs of heterogeneity can be altered by prior in vivo treatment indicating that they express properties of the cells in their in situ configuration. Elevated basal levels of (pro)insulin may reflect the existence of an increased number of beta cells that are activat-

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Abbreviations: GLP-1, Glucagon-like peptide-1.

ed at low physiologic glucose concentrations. Reductions in stimulated insulin levels can be caused by decreased numbers of beta cells that are activated at the prevailing glucose concentration or by insufficient cyclic AMP levels in beta cells, possibly as a result of inadequate signalling from hormones of local or distal origin. Only few markers are currently available with which to explore these mechanisms in vivo. Additional markers and tests should help assess the possible role of variations in beta-cell heterogeneity in the pathogenesis of diabetes mellitus. [Diabetologia (1994) 37 [Suppl 2]: S57–S64]

Key words Beta cells, islets, insulin, diabetes mellitus, pancreas.

New paths of investigation in purified beta-cell preparations

The Minkowski lecture of 1986 reviewed the methods which we developed for the purification of rat beta cells, and illustrated the usefulness of the isolated cell preparations in assessing properties of the insulin-producing cells [1].

The availability of purified beta cells offered a direct approach to mechanisms which influence survival and function of this cell type. We examined the mode of action through which environmental agents interfere with the life of pancreatic beta cells or regulate their activities [1–4]. The model also allowed us to investigate influences from inside the beta-cell population, such as the number of cells, their aggregated state and their functional diversity [1]. These studies led us to describe two critical levels in the generation of beta-cell responses to glucose (Fig. 1).

One important level of regulation was located at the cellular cyclic AMP production, expressing the control by hormones and neurotransmitters [1, 5]. In purified beta cells, glucose-induced insulin release was highly dependent on the presence of peptides which activate cellular adenyl cyclase activity. In freshly isolated islet preparations, locally discharged glucagon was capable of keeping the cellular cyclic AMP levels sufficiently high so as to synergize the glucose signal for release [3, 6]. In vivo, messengers from both local and distal origin were proposed to regulate the beta-cell content in this nucleotide and hence to prepare the cells for a physiologically appropriate response to glucose [1, 5]. For many years, intestinal factors have been considered as potentiators of insulin release following meals [7–9]. Two gut peptides, gastric inhibitory peptide and glucagon-like peptide 1 (GLP-1) [7–37], are thought to mediate this role [10–14]. Both amplify glucose-induced insulin release from isolated islets and from perfused pancreas preparations [9, 11, 15]. The stimulatory action of GLP-1 is observed at physiologic concentrations and associated with an increased adenylcyclase activity [16, 17]. The messages from GLP-1 and from other gut factors are expected to be integrated with those from other tissues [1, 5], resulting in a cyclic AMP signal which sets the amplitude for the secretory re-

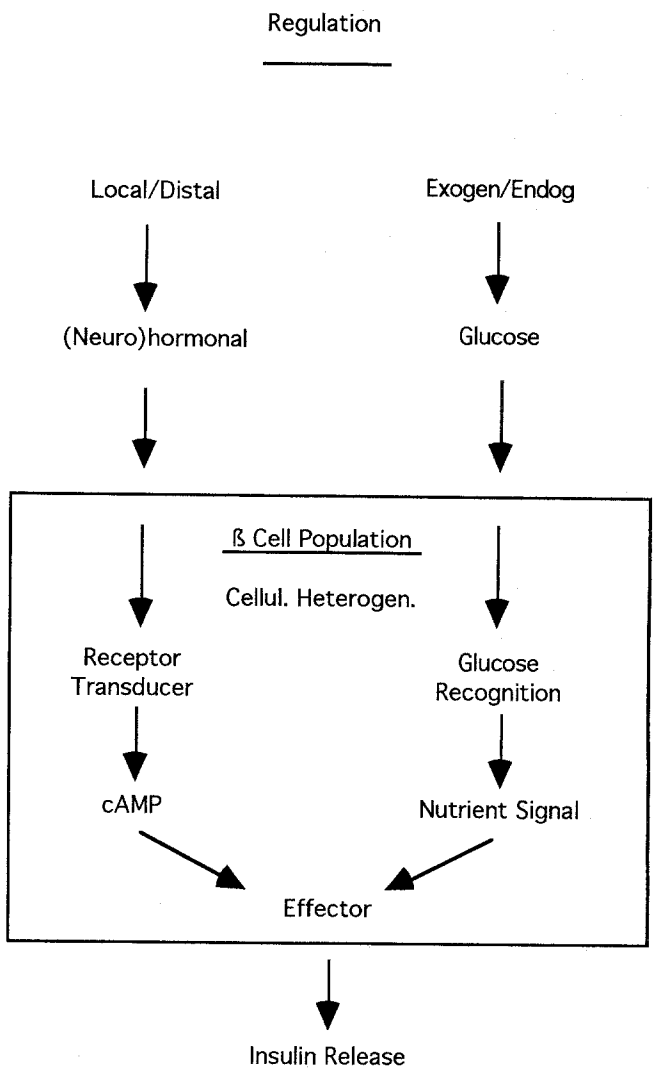

Fig. 1. View on the regulation of insulin release, as proposed in the 1986 Minkowski Lecture [1]. The pancreatic beta-cell population is composed of functionally diverse subpopulations; cellular heterogeneity was noticed in the beta-cell topography towards (neuro)hormonal signals as well as in the cellular glucose recognition. The amplitude of glucose-induced insulin release is determined by a synergistic interaction between the nutrient signal and the cyclic AMP levels which express influences from (neuro)hormonal messengers

sponse to glucose (Fig. 1). An insufficient level of glucose-induced insulin release may therefore not necessarily be caused by failure in glucose recognition by the beta cells [1, 5]. It could also result from inadequate cyclic AMP formation [1, 5], either as a consequence of defects in intracellular signal transduction or of imbalances in the extracellular messages. Such a defect is to be considered in patients with non-insulin-dependent diabetes who release insulin following administration of glucagon or GLP-1 [18, 19] but not – or much less – following injection of glucose. It offers a possibility for GLP-1 receptor agonists to be used in the treatment of certain cases with non-insulin-dependent diabetes [20]. Clinical success along this route would certainly be the best illustration for the physiologic relevance of the model developed from studies on purified cells (Fig. 1).

A second level of regulation was discerned in the functional diversity within the beta-cell population [1]. The marked differences in the properties of individual beta cells were proposed to be complementary in function and hence contribute to the homeostatic control [1]. The observation of intercellular differences in glucose responsiveness [1, 21, 22] led us to a subsequent series of experiments in which the functional consequences and physiologic relevance of this phenomenon were investigated. The present paper reviews the studies which we have undertaken on this subject since 1986.

Beta-cell heterogeneity in glucose responsiveness

Preparations of single and pure beta cells allow comparisons of individual cell activities. The cells were found to exhibit differences in glucose responsiveness using two independent methods of investigation.

In flow cytometry, individual cells were examined for their metabolic redox state at various glucose concentrations [21–23]. Measurement of the cellular NAD(P)H fluorescence intensity monitors the formation of reducing equivalents as a result of glucose metabolism. An increase in NAD(P)H content after 15 min exposure to a particular glucose concentration can be taken as an index for the cell's sensitivity to this nutrient level [21, 22]. The magnitude of the increase will depend on the rate of glucose metabolism as well as on the cellular content in pyridine nucleotides. Marked differences were noticed in the cellular sensitivities to glucose [21–23]. The percent beta cells with elevated NAD(P)H levels increased dose-dependently with the glucose concentration. A rise from 1 to 5 mmol/l recruited 25 % of the cells into metabolic activities; a further increase to 20 mmol/l activated another 30 % of the cells [23]. This dose-dependent recruitment of beta cells into metabolic activity is to be distinguished from an effect of further rises in glucose upon the amplitude of the metabolic response in recruited beta cells.

Autoradiographs of isolated beta cells allowed counting of the percent of cells which were activated into protein synthesis by a particular glucose concentration [24]. Newly-synthesized proteins were labelled with a tritiated amino acid tracer. The fraction of biosynthetically active cells increased dose-dependently with the glucose concentration, from 5 % at 1 mmol/l, to 27 % at 3 mmol/l, 50 % at 5 mmol/l and 70 % at 10 mmol/l. These data demonstrate that intercellular differences are found in the individual biosynthetic activities of beta cells. Glucose dose-dependently increases the number of beta cells which produce new proteins. This glucose-induced recruitment of cells is to be distinguished from amplifications in the rate of biosynthesis which may occur when activated beta cells are exposed to further rises in glucose concentration.

Unpurified islet tissue or cells are less adequate to examine the existence of functional differences among individual beta cells. Besides possible errors in the recognition of beta cells, these beta-cell preparations are under the influence of additional variables, such as the state of cellular aggregation and intercellular communication, the presence of other cell types and of damaged cells. Data obtained from these unpurified cell preparations are nevertheless compatible with the concept of an intercellular heterogeneity in glucose responsiveness. Intercellular differences were noticed in the threshold for glucose-induced electrical activities [25], variations in cytoplasmic calcium [26, 27] and release of insulin [28–31].

The fact that isolated beta-cell preparations are composed of cells with different thresholds for glucose-induced activities strongly suggests that their dose-response curves represent integrations of responses from increasing numbers of activated cells (Fig. 2). Typical characteristics such as low responses at low glucose levels, several-fold stimulation by high glucose levels and dose-dependent variations in the intermediate range of glucose concentrations may require the existence of subpopulations with different glucose sensitivity. Their relative proportions will determine the shape of the curves as well as the amplitude of the glucose response. In the physiologic range of glucose concentrations, the percent activated cells is increased two- to three-fold which may account for a similar rise in glucose-induced functions. Higher rises may result from additional effects of glucose, such as dose-dependent amplification of the nutrient signal in recruited cells. Studies on the total beta-cell population have indicated that the amplitude of glucose-induced insulin release depends on a synergistic interaction between the nutrient signal and the cellular cyclic AMP levels (Fig. 1). It therefore seems conceivable that the rate of insulin re-

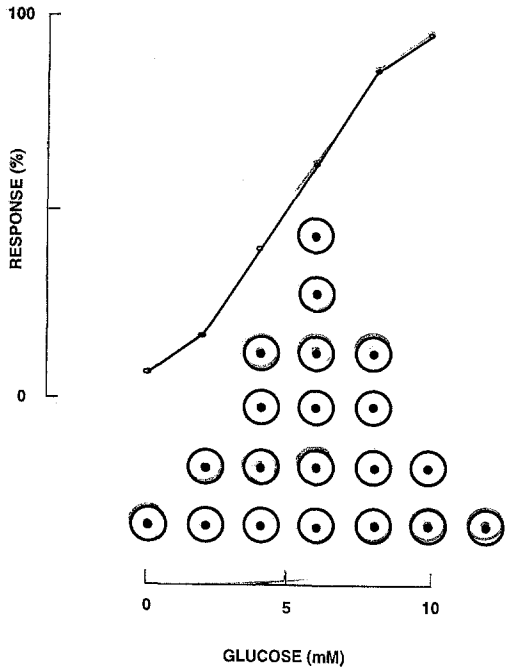

Fig. 2. Effect of cellular heterogeneity on dose-response curves. Model of pancreatic beta-cell population composed of subpopulations with different sensitivities to glucose. A rise in glucose dose-dependently increases the percent activated cells. The shape and amplitude of the dose-response curves are determined by the relative sizes of the activated subpopulations, and by their respective functional capacities

lease is determined by the percent beta cells activated by the prevailing glucose levels and by the cyclic AMP production in these cells generated by interacting hormones and neurotransmittors.

Analysis of functionally heterogeneous beta-cell subpopulations

The flow cytometric detection of beta cells with different sensitivity to glucose was utilized to separate cell subpopulations which differ in their responsiveness to a particular glucose stimulus [21–23]. This separation technique involves sorting of cells according to their NAD(P)H autofluorescence intensity after 15 min incubation at a selected glucose concentration. At 7.5 mmol/l glucose, approximately 50% isolated rat beta cells were recruited into metabolic activities [23]. These cells were separated from the unresponsive cells so that both subpopulations could be compared for their metabolic, functional and morphologic properties.

Virtually all cells that were isolated as metabolically responsive after 15 min exposure to 7.5 mmol/l glucose were biosynthetically activated when subsequently incubated for 60 min at the same glucose level [23]. In contrast, the subpopulation of cells with no or low metabolic responsiveness was poorly recruited during the second incubation at glucose levels up to 7.5 mmol/l. These differences in the respective percentages of biosynthetically activated cells accounted for the different rates of total protein and insulin biosynthesis that were measured in the two subpopulations. Both subpopulations also differed considerably in their secretory responsiveness when perifused, 2–4 h later, at various glucose concentrations [32]. The metabolically responsive subpopulation exhibited a first phase insulin release from 4.2 mmol/l glucose onwards, whereas the unresponsive subpopulation reacted only from 8.3 mol/l. The amplitude of both responses increased dose-dependently with glucose, the rates in the high responsive subpopulation being two-fold higher than those in the low responsive one. The heterogeneity in metabolic and biosynthetic activity is thus associated with a parallel heterogeneity in secretory responsiveness to glucose. It was therefore concluded that glucose dose-dependently recruits beta cells into biosynthetic and secretory activities through a step in its cellular metabolism. In normal rat beta-cell populations, increases in glucose concentration reduce but do not eliminate the percent inactive cells. Co-existence of inactive and activated cells is responsible for a preferential release of newly-synthesized over preformed hormone during glucose stimulation [33].

Sorting according to metabolic responsiveness at 7.5 mmol/l glucose thus separates cells which will or will not become functionally activated during subsequent incubations at glucose levels up to 7.5 mmol/l glucose. Recognition of the step(s) which predispose for this metabolic heterogeneity may thus identify (a) key site(s) in the regulation of glucose-induced functions by the beta cells. We therefore compared glucose metabolism in both subpopulations [34]. The 7.5 mmol/l responsive subpopulation exhibited two- to four-fold higher rates of glucose utilization and oxidation than the unresponsive one. In both subpopulations, the rates of protein synthesis were strongly correlated with those of glucose utilization and oxidation. The higher rates of glucose degradation in the responsive subpopulation were not attributable to higher rates of glucose transport but were associated with higher rates of glucose phosphorylation. The latter ranged also within those of glucose utilization, whereas the rates of glucose transport were at least 50-fold higher. The high responsive subpopulation exhibited a higher glucokinase activity as well as higher glucokinase mRNA levels whereas its GLUT2 mRNA levels were similar to those in the low responsive cells. These data indicate that intercellular differences in glucose phosphorylation – rather than in glucose transport – contribute to the cellular heterogeneity in glucose sensitivity [34]. They are compatible with the view that glucose phosphorylation represents one – but not the sole – key step in the regulation of glucose-dependent functions. They further-

more suggest that mutations in the glucokinase gene may cause diabetes through shifting the distribution of beta cells towards subpopulations with higher thresholds for glucose-induced activation (Fig. 2).

Electron microscopical analysis of the two subpopulations did not reveal any differences in purity or cellular integrity [23]. The cells in the responsive subpopulation were slightly larger and contained a higher hormone content. Since these characteristics may – in themselves – predispose to more insulin release per cell, the rate of secretion is better expressed as percent of the cellular hormone content, at least when the purpose is to compare the secretory responsiveness to a given stimulus [32]. Using a haemolytic plaque assay, a correlation was found between the size of the beta cells and their insulin discharge after maximal glucose stimulation [35] but it remains unknown whether larger cells are more sensitive to glucose. As this technique only measures accumulated hormone discharge per cell, it cannot discriminate between a higher secretory responsiveness and a higher insulin output as a result of higher cellular hormone content or a higher basal discharge [28–31, 35]. If, instead, fractional release rates are measured during perifusion at intermediate glucose concentrations, differences in glucose sensitivity are detected at a higher sensitivity and reliability [32]. The metabolically responsive subpopulation appeared to contain the larger part of newly-synthesized hormone, as judged from their three-fold higher density in pale secretory granules [32]. The proinsulin-rich pale granules seem thus heterogenously distributed over the pancreatic beta cells with the highest density in cells that are sensitive to the lower glucose concentrations. They can thus be taken as a morphologic marker for beta cells which synthesize and secrete hormone at physiologically low glucose levels [33].

Variability in heterogeneity of isolated beta cells

In order to assess whether the pattern of intercellular heterogeneity is variable, we examined whether chronic exposure to high glucose alters the proportions of beta-cell subpopulations with low and high sensitivity to glucose. Isolated rat beta cells were cultured for 10 days at 6 or 20 mmol/l glucose, and then analysed for their individual responses to various glucose concentrations (unpublished observations). After culture at 6 mmol/l, the beta-cell population was still composed of subpopulations with largely different sensitivities to an acute glucose challenge. At 1 mmol/l glucose, less than 20 % of the cells were metabolically or biosynthetically active. Raising the glucose concentration from 1 to 10 mmol/l resulted in a dose-dependent activation of 50 % of the cells. After culture at 20 mmol/l, more than 60 % of the cells were already activated at 1 mmol/l glucose and raising the glucose levels to 5 mmol/l recruited most of the remaining cells into metabolic and biosynthetic activities. These data indicate that the metabolic heterogeneity among beta cells is subject to variation. Glucose qualifies as a physiologic regulator of the proportions of beta-cell subpopulations with different sensitivity to glucose.

Relevance of in vitro model

The concept that heterogeneity in the beta-cell population determines its functional responsiveness to glucose (Table 1) has been exclusively derived from in vitro findings on adult rat beta cells which were purified from isolated islets. That this concept may also apply to the in vivo situation is suggested by the following experiments.

We first examined whether the purified cell preparations can be considered as a representative sample of the pancreatic beta-cell population. Since these were isolated from islets which had been selected by their larger size, first during elutriation and then during hand-picking [36], it was necessary to examine whether their pattern of heterogeneity was also valid for the discarded beta cells, i.e. those present in smaller islets and representing approximately one third of the initial beta-cell mass [36]. Beta cells were therefore purified from both the small and the larger islet structures, using a combination of elutriation gradient centrifugation and cell sorting (method to be published elsewhere). The patterns of metabolic and biosynthetic heterogeneity were superimpossible for all purified beta-cell preparations, i.e. those isolated from hand-picked islets and those prepared without hand-picking from the small or large islet structures. It could thus be concluded that the previously obtained data were representative for the entire beta-cell population. The data also validate an isolation procedure without hand-picking, which was used for the second part of this study.

It was then investigated whether the in vitro pattern of heterogeneity can vary with the in vivo conditions to which the beta-cell population has been exposed prior to its isolation. We chose to test the effects of a two-day treatment with glibenclamide at concentrations which were previously shown to degranulate the beta cells and enhance their gap junctional communication [37]. When isolated, the beta-cell population from glibenclamide-treated animals was found to contain larger proportions of cells that were already activated in the absence of glucose; furthermore, virtually all of the remaining cells were recruited by glucose [38]. This altered pattern of heterogeneity was associated with a shift in the dose-response curves towards higher rates of insulin synthesis, in particular in conditions with or without low glucose concentrations [38]. These data show that in

Table 1. Role of beta-cell heterogeneity in glucose homeostasis

Regulation
↓
Intercellular differences in glucose metabolism (marker = glucokinase)
↓
Intercellular differences in glucose-induced biosynthesis and release (marker = pale granules)
↓
Dose-response curves of total beta-cell population (marker = release of newly formed hormones)

vivo conditions influence the pattern of heterogeneity which is observed during analysis of the isolated beta-cell population. As with all in vitro studies, it cannot be excluded that the isolation procedure modulates – in itself – certain in vivo characteristics. For this reason, the heterogeneity in the beta-cell population should also be examined in situ.

Beta-cell heterogeneity in the intact pancreas

The pancreatic beta-cell population also exhibits signs of functional heterogeneity in its in situ location. In perfusion studies on the rat pancreas, glucose dose-dependently stimulates insulin release over a similar concentration range as in purified beta-cell preparations [39]. The intact pancreas may thus also be composed of beta-cell subpopulations with different sensitivities to glucose. This was assessed by counting the percent beta cells in active biosynthesis during perfusion at different glucose concentrations with ^{3}H leucine as marker for newly-synthesized proteins. This percentage increased dose-dependently, with virtually all beta cells labelled at 10 mmol/l glucose [24]. In adult rats, prolonged glucose stimulation did not lead to the same degree of degranulation in all beta cells [40], suggesting differences in cellular responsiveness or in the initial state of degranulation. In untreated animals, the intensity of the insulin immunostaining varies considerably among beta cells; most cells are weakly positive for proinsulin but a few exhibit an intense positivity [41]. This variability in hormone staining is also noticed in dispersed islet cell preparations; the stronger intensity in beta cells that are structurally coupled to somatostatin-containing cells [1] suggests topographic differences in functionally diverse beta cells. In situ heterogeneity was also found for the proliferative activity of the beta cells, with only a small proportion of cells involved in DNA synthesis [42].

The sequence of events which was derived from studies on purified rat beta cells has not yet been documented in vivo. Current data support the existence of a beta-cell heterogeneity in the intact pancreas and its variability under influence of well-known beta-cell tropic agents such as glucose and sulphonylurea. Knowledge about the mechanisms which are operative in vitro may provide markers for the detection of these processes in vivo. In vivo analysis is necessary to further examine the hypothesis that the heterogeneity among beta cells plays an important role in the physiology and pathology of the endocrine pancreas [33, 43].

Markers for analysing beta-cell heterogeneity in situ

The analysis of beta-cell heterogeneity in situ would be facilitated if markers were available which identify – in the intact organ or organism – the activated state of the cells, in particular for their metabolic, biosynthetic and secretory activities (Table 1). There are presently three markers which can be useful in this respect.

Glucokinase regulates the rate of glucose metabolism in beta cells and, consequently, that of glucose-induced functions [44, 45] (Table 1). The enzyme is expressed more strongly and is more active in beta cells with high sensitivity to glucose [34]. It may, therefore, qualify as a marker for the cellular glucose sensitivity if the protein and its activity can be quantified in individual cells or cell subpopulations. Using immunocytochemistry on islet sections, Jetton and Magnuson [46] detected intercellular differences in glucokinase immunoreactivity, adding support to the existence of a metabolic heterogeneity among beta cells. It will be interesting to correlate this heterogeneity in immunostaining with the metabolic properties of the cells so that this in situ analysis of cellular heterogeneity can be interpreted in physiologic terms.

Pale granules were more abundant in the beta-cell subpopulation with sensitivity to low glucose levels [23]. A high cellular density in this type of granule may mark the beta cells which are already activated at basal glucose levels [33]. When determined in intact pancreatic tissue, the percent beta cells with a high absolute and relative content in pale granules may indicate the size of the high responsive subpopulation. It is then expected that conditions with increased proportions of this subpopulation will generate increased rates of insulin release at basal glucose levels. To our knowledge, this type of analysis has not yet been performed on individual beta cells of intact tissue. Previous studies have, however, counted the ratio of pale over total granules in the beta-cell area. Increased ratios were found in conditions with increased basal insulin levels such as in animals undergoing cortisone treatment [47] or with a growth hormone producing tumour [48], in obese [49] and pregnant [50] rats. The insulin-containing cells of an insulinoma are also characterized by a high content

of pale granules. It seems likely that further analysis of these conditions will demonstrate that the higher basal activities are indeed generated by the presence of increased numbers of beta cells with high density of pale granules. The counting and typing of granules in a sufficient number of beta cells remains, however, a tedious technique, despite the availability of image analysers. Until easier markers become applicable, the pale granule can be used as an index for high responsive beta cells.

The release of newly-formed insulin expresses the activity of beta cells which have been recruited into biosynthetic and secretory activities [32, 33]. The beta-cell population contains a subpopulation which is activated at low glucose levels and which is characterized by a higher content of pale proinsulin-rich granules [33]. It is thus conceivable that the circulating proinsulin levels at basal glucose concentrations reflect the activity of the high responsive beta-cell subpopulation. Conditions with an increased size of this subpopulation may therefore be associated to higher basal proinsulin levels. Serum proinsulin could thus represent an easily accessible marker for the size of the high responsive beta-cell subpopulation. It is, of course, known that the circulating proinsulin levels are also influenced by factors other than the activity of the pancreatic beta cells, which raises the need for developing additional functional tests which specifically evaluate the high responsive cells.

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