

Human Type 2 Diabetes

Morphological Evidence for Abnormal β -Cell Function

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The exact nature of the β -cell defect in type 2 diabetic patients is still unclear. β -Cell mass reduction has been reported but remains controversial. A preliminary study of a large series of patients has demonstrated that in most, the β -cell defect is not related to a decreased β -cell mass. Amyloid deposits are observed in the islets of some type 2 diabetic patients but also in normoglycemic subjects. Because it has been claimed that these deposits interfere with β -cell function, we evaluated in situ the effect of insular amyloid deposits on β -cell transcription and translation. Pancreases were obtained at autopsy from 28 normoglycemic patients and 41 type 2 diabetic patients. Staining with hemalun-eosin and Congo red was used to analyze the general features of the islets and the presence of amyloid deposits, respectively. Immunohistochemistry for proinsulin was performed with an antibody recognizing the junction between B-chain and C-peptide, thus specifically labeling the Golgi area where proinsulin is produced. In seven patients, we evaluated insulin gene transcription by in situ hybridization of proinsulin mRNA combined with Congo red staining, and we evaluated insulin storage by double immunostaining for insulin and amylin. In many type 2 diabetic patients, the islets appeared entirely normal. Amyloid deposits were found in 57% of diabetic subjects and 33% of normoglycemic age-matched control subjects. The percentage of amyloid-infiltrated islets varied from 0.4 to 74%. β -Cells from amyloid-containing islets still had specific Golgi proinsulin labeling. In obese type 2 diabetic patients, the number of β -cells with abnormal expression of proinsulin in the whole cytoplasm was significantly higher than in normoglycemic control subjects. Proinsulin mRNA was significantly reduced in islets with amyloid deposits when compared with amyloid-free islets, but the mean reduction did not exceed 16%. Insulin was still present in the β -cells of amyloid-containing islets, and its amount, estimated by measurement of the insulin-labeling optical density, was not statistically different from that in amyloid-free islets. In conclusion, even in amyloid-containing islets, β -cells maintain active insulin transcription and translation and normal insulin storage. Taking into account that in most cases only a small proportion of islets are infil-

trated by amyloid, the limited reduction in proinsulin mRNA is unlikely to play a major role in the pathogenesis of diabetes. *Diabetes* 50 (Suppl. 1):S172–S177, 2001

The pathogenesis of type 2 diabetes remains controversial. Although there is general agreement that both insulin resistance and a β -cell defect contribute to the disease, the relative importance of each factor, the primary event, and the nature of the β -cell dysfunction are still unclear (1,2). A sufficient mass of β -cells is required to ensure normal glycemic regulation; indeed, transplantation experiments demonstrate that enough islets must be grafted to restore normoglycemia (3), and hyperglycemia does not occur in type 1 diabetes as long as residual β -cell mass remains >30–50% (4–6). Also, the capacity of β -cells to synthesize and secrete insulin has to be preserved: in hemochromatosis the number of islets is normal, but insulin synthesis seems to be inhibited (7) when patients develop secondary diabetes.

The impressive image of pancreatic islets destroyed by amyloid deposits (Fig. 1A) and the clinical experience that type 2 diabetic patients may eventually require insulin have often been linked, resulting in the widespread idea that type 2 diabetes results from reduction of β -cell mass. Several studies have suggested that β -cell mass is decreased, but these remain controversial (8–15), owing to technical factors such as sampling, staining, and quantification methods. Thus, it remains unclear whether there is real β -cell loss in type 2 diabetes.

We have recently extended our initial study (13) to a large series of type 2 diabetic patients whose BMI and mode of treatment were taken into account. Preliminary results show that, even after many years of diabetes leading to complications, more than 80% of type 2 diabetics have a β -cell mass within the range of that in control subjects (16). Thus, in most patients, insufficient insulin secretion is not caused by decrease in β -cell mass. Although not present in all cases—or in all islets in the same patient—amyloid deposits have often been considered the hallmark of type 2 diabetes (17). Because amyloid fibrils are closely linked to the β -cell membrane (Fig. 2), it has been hypothesized that the membrane of β -cells in amyloid-containing islets is altered and that their function is markedly impaired (17). However, this interesting hypothesis has not yet been tested because the functional state of β -cells has seemed difficult to assess on postmortem fixed and paraffin-embedded material. The aim of the present study was to analyze insulin transcription and translation in amyloid-containing and amyloid-free islets and to evaluate whether their alteration could contribute to the pathogenesis of type 2 diabetes.

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IAPP, islet amyloid polypeptide; ISH, in situ hybridization.

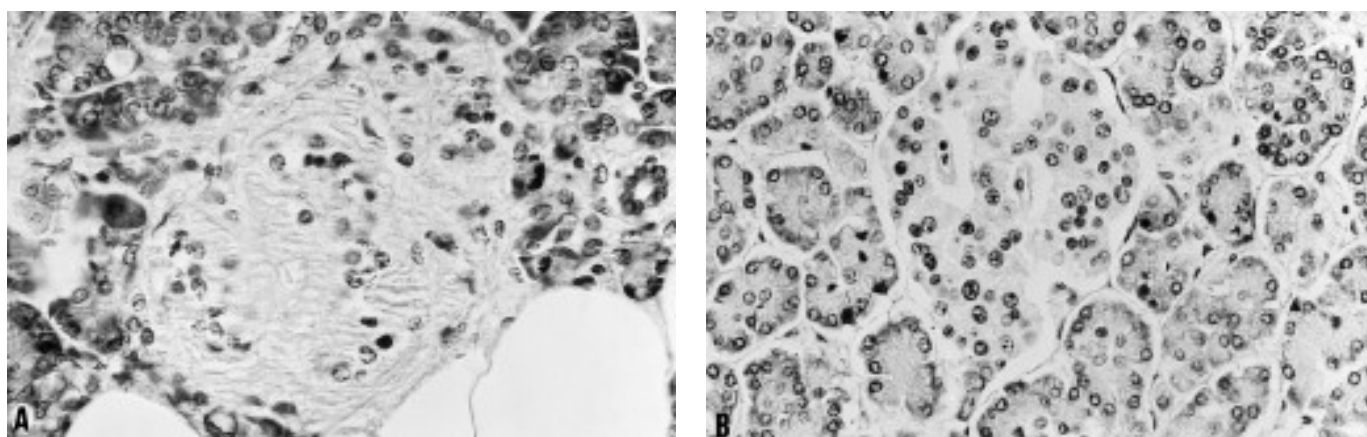


FIG. 1. *A*: Large amyloid deposits in a pancreatic islet of a type 2 diabetic patient (Congo red staining, $\times 365$). *B*: Islet of Langerhans may also be of strictly normal appearance in type 2 diabetic patient (hemalun-eosin, $\times 365$).

RESEARCH DESIGN AND METHODS

Pancreases were obtained at autopsy performed within 12 h after death from 28 normoglycemic and 41 type 2 diabetic patients. Two specimens (3–4 mm thick) from each region of the pancreas were fixed in either 4% buffered paraformaldehyde or Bouin's solution before being embedded in paraffin. Four consecutive sections, 5 μ m thick, were cut from the body of the gland and stained with hemalun-eosin and Congo red to identify even small deposits of amyloid. Insulin and proinsulin were detected on successive sections by indirect immunoperoxidase methods using anti-insulin antibody (HUI-018, NovoClone) and proinsulin antibody (HUI-005, NovoClone) at dilutions of 1/2,000 and 1/500, respectively, and 3,3'-diaminobenzidine hydrochloride as substrate. The proinsulin antibody recognizes the junction between the B-chain and C-peptide and thus labels both intact proinsulin and the proinsulin conversion intermediate des-64,65-proinsulin. Proinsulin immunodetection assesses both active prohormone synthesis and its cleavage into insulin and C-peptide. Thus, when proinsulin is processed normally, the labeling is restricted to the site of synthesis, the Golgi area, whereas abnormal proinsulin processing results in a more diffuse labeling over the whole cytoplasm. To evaluate abnormalities in proinsulin cleavage, β -cells with labeling of the whole cytoplasm were counted in a whole transverse section of the pancreas and expressed per 100 mm^2 of pancreatic tissue.

The pancreases from seven patients obtained at autopsy within 5 h after death were used to evaluate insulin gene transcription and β -cell insulin content. In situ hybridization (ISH) for insulin mRNA was performed on formalin-fixed and paraffin-embedded pancreases (18) and was combined with Congo red staining for amyloid. A double immunohistochemical method was used to demonstrate simultaneously insulin in brown, with 3,3'-diaminobenzidine hydrochloride as substrate of peroxidase, and islet amyloid polypeptide (IAPP) (with anti-IAPP antibody IHC-7321 from Peninsula Laboratories at 1/2,000) in red, with naphthol and fast red as substrate for alkaline phosphatase. These seven cases were treated simultaneously to ensure homogeneity of technique. Densitometric measurements of the intensity of insulin and insulin mRNA labeling were performed with a computerized image analyzer (Ibas, Kontron, Germany); details about the method and its reliability have been reported previously (19). All selected cases contained islets with and without amyloid deposits. In each patient, insulin mRNA and insulin storage were quantified in 20 islets of both types. The relative area of amyloid deposits was measured in each islet with an image analyzer and was correlated with insulin mRNA estimated by densitometry.

RESULTS

General features. After hemalun-eosin staining, the appearance of islets was quite variable between type 2 diabetic patients and between islets from the same patient. Many islets were of strictly normal appearance (Fig. 1*B*), whereas others were infiltrated by amyloid deposits (Fig. 1*A*). In this series, amyloid was found in 57% of diabetic patients but also in 33% of normoglycemic age-matched control subjects.

The presence of amyloid was not related to diabetes duration or to obesity. In diabetic patients with amyloid deposits, not all islets were infiltrated. The range was broad (0.4–74%), with 80% of the patients having <20% of their islets infiltrated with amyloid (Fig. 3). Amyloid was present in >40% of the islets in only 6% of the patients. In nondiabetic patients with amyloid, the proportion of infiltrated islets averaged 11%, with a range from 0.7 to 32%. Many islets also appeared entirely normal after insulin labeling (Fig. 4*A*). Islets containing amyloid, sometimes even in large amounts, still had a high number of insulin-containing cells (Fig. 4*B*). Conversely, in certain islets devoid of amyloid, β -cells were no longer the predominant cell type (Fig. 4*C*). This alteration of the interrelationship between endocrine cells was observed in up to 10% of the islets; it was mostly, but not exclusively, observed in diabetic patients.

Insulin gene expression. Proinsulin mRNA was easily demonstrated in all cases where autopsy was performed within 5 h after death, but labeling intensity varied between cases. On the same sections, amyloid was easily recognized after Congo red staining. Proinsulin mRNA labeling was positive in both amyloid-containing and amyloid-free islets, but

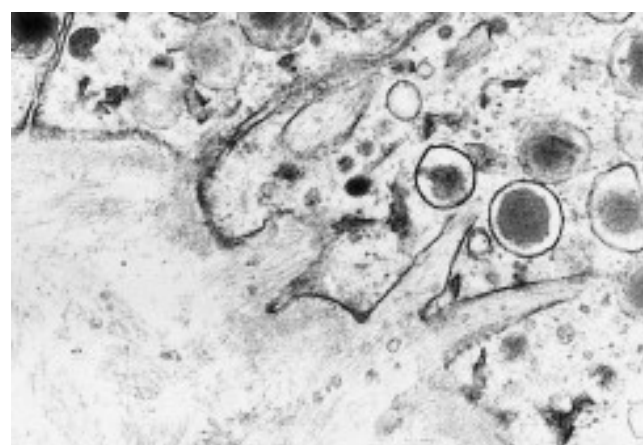


FIG. 2. Amyloid fibrils closely located at the β -cell membrane (electron microscopy, $\times 170,000$).

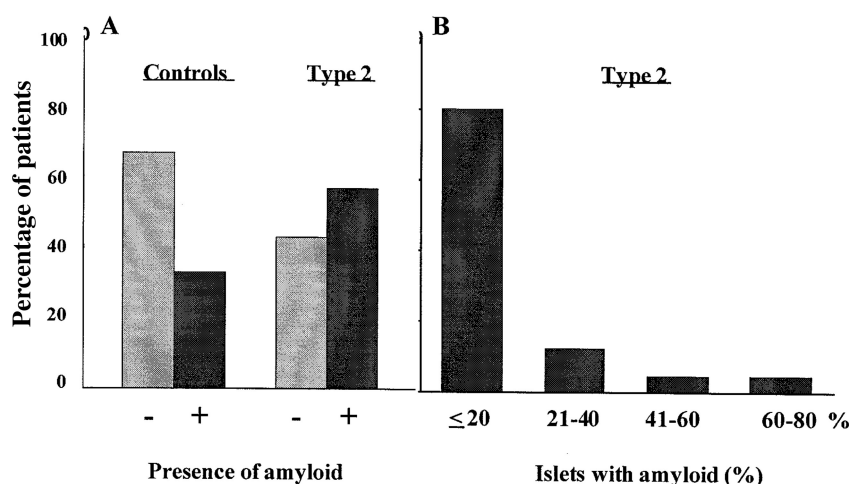


FIG. 3. A: Percentage of normoglycemic control subjects and type 2 diabetic patients with and without pancreatic amyloid deposit. B: Distribution of the relative percentage of amyloid-infiltrated islets in type 2 diabetic patients.

appeared slightly less intense in the former (Fig. 5); indeed, densitometry of insulin mRNA showed a consistent decrease (by $16 \pm 4\%$, mean $\pm$ SD; $P < 0.02$) in amyloid-containing islets versus amyloid-free islets from the same patient (Fig. 6). No correlation was found between the amount of amyloid within the islet and the reduction of insulin gene expression.

Proinsulin mRNA translation and proinsulin processing.

Staining of the β -cell Golgi area by proinsulin antibody was not different in diabetic patients and normoglycemic control subjects. β -Cells within amyloid-containing islets still had specific Golgi proinsulin labeling (Fig. 7A), even when the deposits were large. In some β -cells, staining for proinsulin gave a picture similar to that usually detected with the insulin antibody, labeling of the whole cytoplasm, reflecting poor cleavage of the prohormone (Fig. 7B). The number of β -cells with uncleaved proinsulin in the whole cytoplasm was higher in type 2 diabetic patients than in normoglycemic control subjects ($P < 0.001$). However, when diabetic subjects were subdivided into lean and obese patients, another difference emerged. An increased proportion of β -cells with abnormal proinsulin processing was prominent in obese patients ($P < 0.001$), but there was no difference between lean patients and control subjects (Fig. 8). β -Cells with uncleaved proinsulin represented up to 18% of all β -cells in obese type 2 diabetic patients.

Insulin storage. The intensity of insulin immunolabeling per β -cell area was similar in diabetic patients and control subjects, and it did not seem different in amyloid-containing and amyloid-free islets; densitometric measurements of insulin immunolabeling confirmed the absence of difference between the two groups.

DISCUSSION

The disparity of histological alterations in islets in type 2 diabetes is not understood. Contrary to a widespread idea, it is unlikely that amyloid is simply related to diabetes duration, since it is sometimes observed after short duration of disease but may be absent after a long evolution. It is not related to obesity, because it is observed with the same frequency in obese and in lean patients. Because the mechanism of amyloidogenesis is still unknown, it is difficult to understand why amyloid is present in only certain patients and in these, usually in a minority of islets. The fact that amyloid was also

observed in islets of 33% of normoglycemic control subjects and was absent in 43% of diabetic patients suggests that amyloid infiltration is not the primary event in the development of type 2 diabetes. Studies on the potential role of amylin, the major constituent of amyloid in type 2 diabetes, in β -cell function failed to demonstrate inhibition of insulin secretion in humans (20) or of insulin gene expression, proinsulin synthesis and conversion, and insulin secretion in cultured rat islets (21). It is thus unlikely that the slight decrease in proinsulin mRNA that we observed in amyloid-containing islets is the consequence of an effect of amylin on β -cells. Interestingly, hyperexpression of human IAPP in transgenic mice induced both amyloid deposits and diabetes, the latter apparently preceding the formation of amyloid deposits (22). In this model, several arguments suggest that newly formed intermediate-sized amyloid particles could be toxic for the β -cell membrane and lead to cell death (23). Major atrophy of the islets is observed in this model, which is thus markedly different from human type 2 diabetes.

The occurrence of islets in which β -cells cease to be the predominant cell type is not specific to diabetes; it is observed in control subjects as well. Such islets were never observed in children or young adults and thus are likely to be related to aging. The amount of insulin mRNA greatly varied between patients. This is probably the consequence of differing premortem conditions and treatments. On the other hand, the differences between amyloid-containing and amyloid-free islets are real because comparisons were made within each pancreas. Densitometric measurement of ISH labeling unequivocally demonstrated that the presence of amyloid deposits is associated with lower proinsulin mRNA content. This study does not permit us to conclude whether this decrease results from direct or, as previously suggested, indirect effects (23,24). The impact of this decrease in proinsulin mRNA on insulin secretion is probably negligible: on average it did not exceed 16%, and the percentage of islets with amyloid was only 13%. Thus, it may be concluded that the presence of amyloid, even in large amounts, only marginally affects insulin gene expression.

Proinsulin immunoreactivity was detectable in β -cells from control and diabetic patients. The antibody used recognizes both intact proinsulin and des-64,65-proinsulin. Because the latter is a minor conversion intermediate (25), it is likely that the antibody mainly demonstrates intact proin-

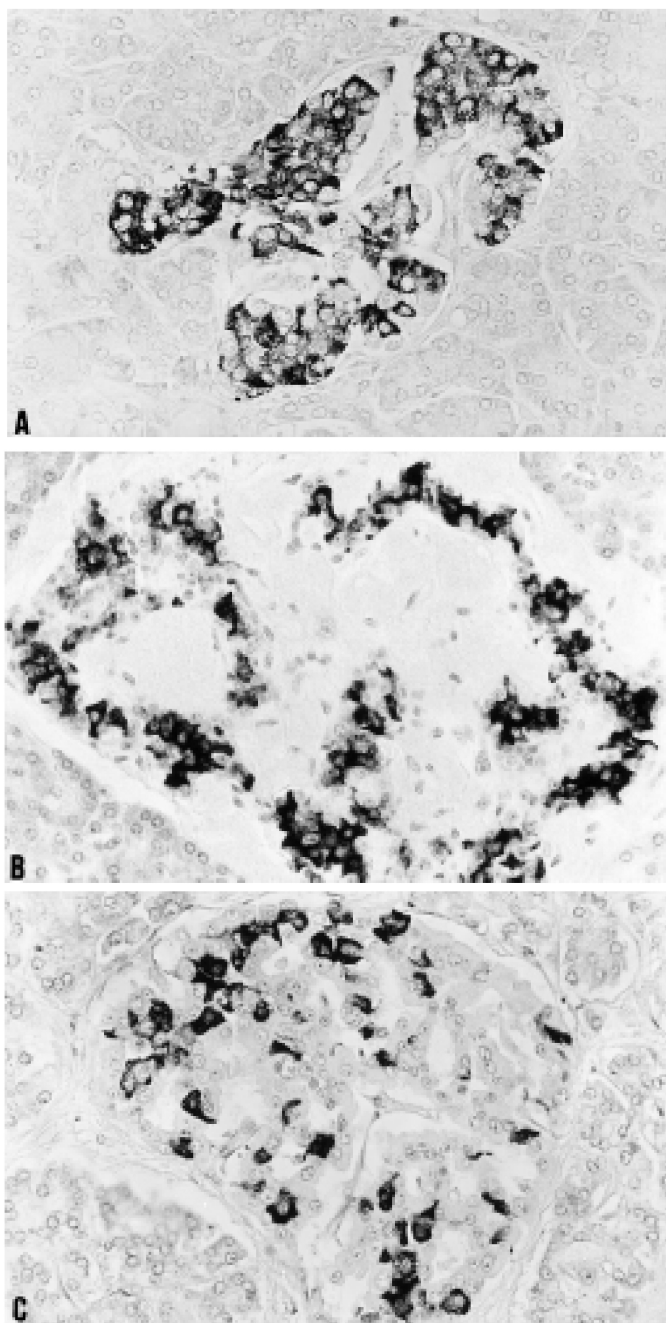


FIG. 4. Even in the presence of large amyloid deposits, β -cells still contain large amounts of immunoreactive insulin in a normoglycemic control subject (A) and a type 2 diabetic patient (B). In some type 2 diabetic islets, β -cells are not the predominant cell type (C) ($\times 190$).

sulin. This explains why it labels essentially the Golgi area in normal β -cells. In amyloid-containing islets from type 2 diabetic patients, proinsulin immunoreactivity was still detected in the Golgi area. Although it has been claimed that β -cell function in amyloid-containing islets is impaired and that such β -cells should therefore not be taken into account when evaluating the β -cell mass (17), the presence of both proinsulin mRNA and proinsulin in the Golgi area suggests that these β -cells are still active at both the transcriptional and the

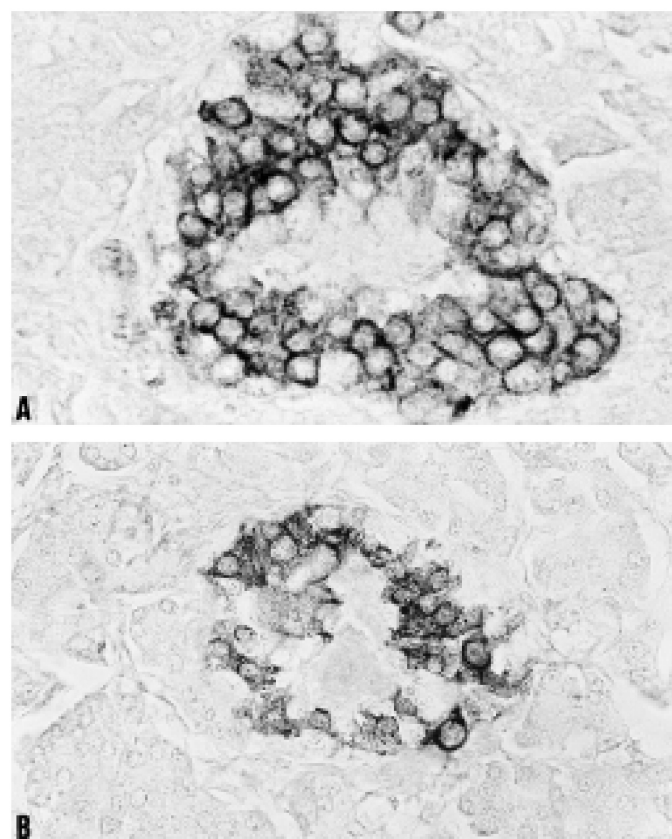


FIG. 5. In situ proinsulin mRNA detection in an amyloid-free (A) and an amyloid-containing (B) islet ($\times 650$). Amyloid deposit is localized in the core of the islet.

translational level. Certain β -cells, mainly in obese type 2 diabetic patients, showed inadequate processing of proinsulin, because labeling was not restricted to the Golgi area but was found in the whole cytoplasm. A high proinsulin-to-insulin ratio in the circulation has been repeatedly reported in type 2 diabetes (26–28). Our results demonstrating a high proportion of cells with uncleaved proinsulin in obese type 2

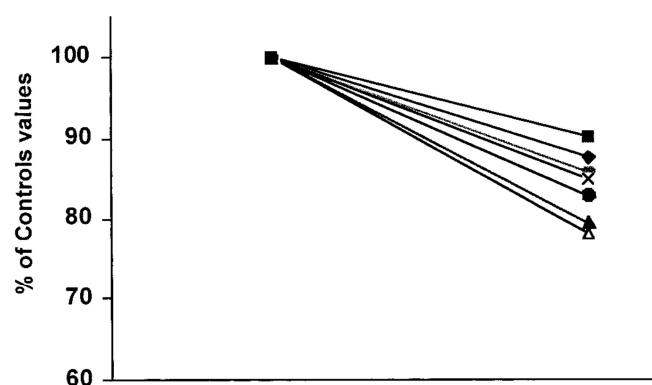


FIG. 6. Optical density values of proinsulin mRNA in amyloid-free and amyloid-containing islets. For each patient, results are given as a percentage of control value, which is the mean intensity of proinsulin mRNA staining in amyloid-free islets ($P < 0.01$, paired Wilcoxon's rank-sum test).

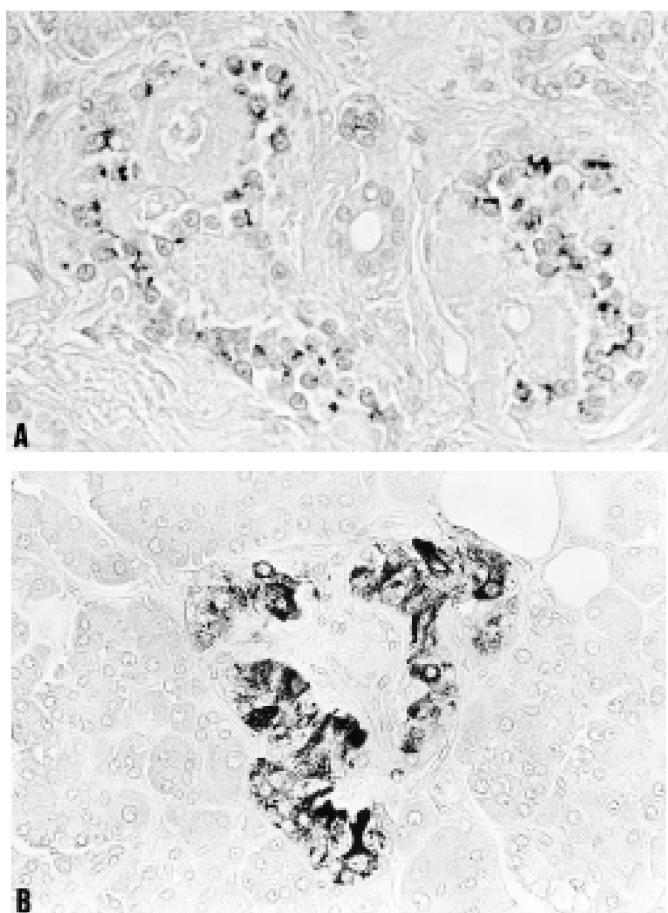


FIG. 7. Immunohistochemical staining of proinsulin is restricted to the Golgi area in normal β -cells. In islets with large amyloid deposits, proinsulin staining may still be normal (A). In some β -cells from type 2 diabetic patients, proinsulin labeling is observed in the whole cytoplasm, reflecting abnormal proinsulin processing (B) ($\times 365$).

diabetic patients but not in obese normoglycemic control subjects fit particularly well with those of Røder et al. (28) and suggest that hyperproinsulinemia is due to β -cells with abnormal proinsulin cleavage. It remains unclear whether these β -cells constitute an abnormal population or whether all β -cells are susceptible to this defect.

Finally, because densitometric insulin measurements did not reveal differences between islets with and without amyloid and because the intensity of insulin labeling was not different in normoglycemic and diabetic patients, it is likely that the regulation of insulin storage is not affected in type 2 diabetes, even in islets infiltrated by amyloid.

Our preliminary study concluding that the β -cell mass is not significantly diminished in most type 2 diabetic patients and the demonstration that β -cells maintain active insulin gene transcription and translation even in amyloid-containing islets and that insulin cleavage is normal in most β -cells suggest that the major problem in type 2 diabetes is abnormal coupling of insulin secretion to glycemia.

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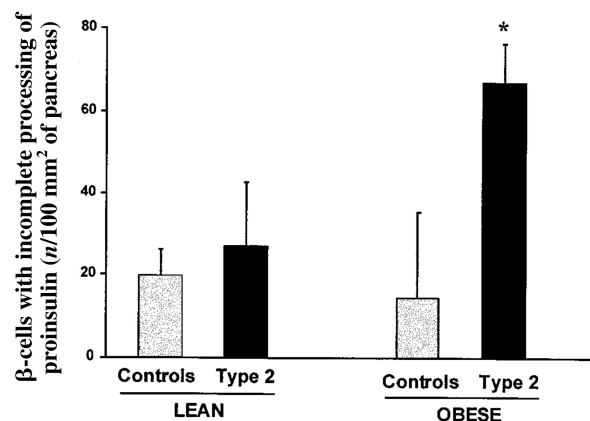


FIG. 8. β -Cells with uncleaved proinsulin in whole cytoplasm were counted in the whole section and referred to pancreatic area in lean or obese type 2 diabetic patients and in normoglycemic control subjects. Results are expressed as means $\pm$ SE. * $P < 0.001$, type 2 diabetic patients vs. all other groups.

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