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Analysis of pancreatic islet cells and hormone content in the spontaneously diabetic KKAy mouse by morphometry, immunocytochemistry and radioimmunoassay

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Summary. The splenic pancreas of 165 day old diabetic KKAy and age-matched nondiabetic C57BL/6 mice was examined by morphometry and immunocytochemistry at the light microscopic level and by radioimmunoassay to evaluate the morphology, surface area, endocrine cell composition and hormone content of the pancreatic islets. The insulin cells of the diabetic mice were severely degranulated and many of the glucagon, somatostatin and pancreatic polypeptide cells were displaced from the mantle to the core of the islet tissue where the non-insulin cells appeared to lose their continuity. The topography of some of the islets of KKAy mice was further deranged by acinar cells among the endocrine tissue. Morphometric analysis revealed that the surface area of the islets of KKAy mice was significantly expanded in comparison with that of C57BL/6 mice. The volume and numerical percents of the insulin cells were significantly increased whereas those of the glucagon and somatostatin cells were decreased in the KKAy mice. Since only the mean absolute number of insulin cells was elevated in the diabetic mice, the alteration in the relative proportions of the non-insulin cells and hypertrophy of the islets seemed to be a manifestation of insulin cell hyperplasia. Pancreatic insulin and somatostatin contents were markedly diminished in the islets of KKAy compared with those of C57BL/6 mice. These results demonstrate that the microscopic anatomy, endocrine cell populations and hormone content of the pancreatic islets are deranged in the KKAy mouse with severe hyperinsulinemia and hyperglycemia.

Key words: KKAy mice – Pancreatic islets – Morphometry – Immunocytochemistry – Radioimmunoassay

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Introduction

A genetic colony of yellow obese, diabetic KKAy mice has recently been established at The Upjohn Company for the purpose of in vivo evaluation of novel antidiabetic compounds that may ameliorate hyperglycemia in Type II (noninsulin-dependent) diabetic patients and/or prevent or retard complications such as nephropathy. Original breeding stock consisting of yellow KKAy males and black KK females were procured from Takeda Chemical Industries, Osaka, Japan. Information relative to the breeding scheme and genetic background of the KKAy mouse has been reported (Iwatsuka et al. 1970).

Due to the fact that the KKAy mouse carries yellow obese and diabetes genes, this diabetic animal model displays early onset and prolonged, severe hyperinsulinemia and hyperglycemia (Iwatsuka et al. 1970; Chang et al. 1986). It is important to point out that the genetic composition and metabolic characteristics of the KKAy mouse are dissimilar to those of the KK mouse, since the latter animal possesses only the diabetes gene and exhibits hyperinsulinemia and glucose intolerance but not overt hyperglycemia (Iwatsuka et al. 1970). Although the metabolic defects (Chang et al. 1986) and renal glomerular capillary basement membrane thickening (Diani et al. 1987) of the KKAy mouse have been adequately described, there has been no effort to systematically elucidate the alterations in the pancreatic islets of this animal model. Since derangement of pancreatic islet morphology, endocrine cell populations and hormone content has been demonstrated in obese, hyperglycemic, hyperinsulinemic mutant mice such as the ob/ob (Coleman and Hummel 1973; Baetens et al. 1978; Patel et al. 1977; Gingerich et al. 1978; Dolais-

Kitabgi et al. 1979; Makino et al. 1979) and db/db (Coleman and Hummel 1967; Like and Chick 1970; Boquist et al. 1974; Patel et al. 1977; Baetens et al. 1978; Makino et al. 1979; Leiter et al. 1979), it would be interesting to determine if comparable abnormalities are evident in the islets of KKAy mice. Therefore, the purpose of this investigation is to evaluate pancreatic islet morphology, surface area and endocrine cell composition by morphometry and immunocytochemistry at the light microscopic level and hormone content by radioimmunoassay of the splenic pancreas of 165 day old diabetic KKAy compared with age-matched nondiabetic C57BL/6 mice.

Materials and methods

Animals. Diabetic KKAy mice at 165 days of age and nondiabetic age and sex-matched C57BL/6 mice were obtained from The Upjohn Company colonies for this investigation. Each mouse was fed Purina Mouse Breeder Chow 5015 and water ad libitum and maintained in an individual cage under controlled photoperiod (12 hours light/day; lights on at 6 a.m.). Each mouse was terminated by exsanguination and the blood (nonfasted) was assayed for glucose (Lloyd et al. 1978) glycosylated hemoglobin (Glycoglobin Kit, Endocrine Sciences Products, Inc., Tarzana, CA, USA), plasma insulin (Zaharto and Beck 1968) and plasma glucagon (Faloona and Unger 1974).

Study 1

Light microscopy and morphometry (aldehyde-fuchsin and immunocytochemical staining) of pancreatic islets. Immediately after termination the splenic pancreas of KKAy ($n=11$) and C57BL/6 ($n=10$) mice was immersion-fixed in Bouins for 4 h, dehydrated in an ascending series of ethanols and embedded in paraffin. At 200 μm intervals, 5 serial sections (5 μm each) were cut on a rotary microtome and adhered to individual glass slides with poly-L-lysine.

The first section from each series was stained with Gomori's aldehyde-fuchsin and each islet within that section was subsequently examined under brightfield light microscopy to evaluate the granulation of beta cells and morphology of the islet tissue. The percent incidence of acinar cell displacement into the pancreatic islets was obtained for each KKAy and C57BL/6 mouse. Morphological analysis was conducted on an average of 41 (range 18–69) islets per KKAy mouse and an average of 33 (range 11–43) islets per C57BL/6 mouse. The aldehyde-fuchsin sections were also projected by a microprojector ($\times 450$) onto the digitizing tablet of a Zeiss Videoplan Image Analysis System. The surface area (μm^2) of each islet within a section was measured by tracing the periphery of the endocrine tissue with a cursor. Pancreatic islets with 8 or more cell nuclei were measured. Acinar cell displacement into some of the pancreatic islets of KKAy mice severely disrupted the anatomical integrity and boundaries of the endocrine tissue. Therefore, islets with acinar cells were not measured. Morphometric analysis was carried out on an average of 35 (range 14–60) islets per KKAy mouse and an average of 33 (range 11–43) islets per C57BL/6 mouse. Due to excessive variability among the islet measurements of both groups of mice, a log transformation of the data was performed. All observations and measurements were made by an individual unaware of animal identity.

The remaining 4 sections in each series were consecutively immunostained with polyclonal primary antibodies against insulin, glucagon, somatostatin and pancreatic polypeptide according to the unlabelled peroxidase anti-peroxidase protocol of the Tissue-Tek® Immunohistology Kits (Miles Scientific, Naperville, IL, USA) for insulin (Kit 8652), glucagon (Kit 8650), somatostatin (Kit 8665) and pancreatic polypeptide (Kit 8620). The only deviation from the protocol described in each of the Immunohistology Kits was a 16 h incubation (instead of 20 min) with the primary insulin antibody of the sections from the KKAy mice. This alteration provided a more intense stain of the degranulated insulin cells of the diabetic mice to insure accurate identification for morphometric analysis. All sections were incubated with the chromogen aminoethylcarbazole to provide a red, water insoluble precipitate for precise identification of each antigen (hormone). All sections were counterstained with Mayer's hematoxylin to permit quantification of islet cell nuclei. Control sections (Sternberger 1974) were also included in the staining procedure for each pancreatic islet hormone. The immunostained pancreatic islets were projected by a microprojector ($\times 450$) onto a 5 mm^2 grid. The total number of points which hit the islet, as well as those which touched the cytoplasm and nucleus of the immunostained cells, was obtained. The percent volume of each type of endocrine cell was generated as the ratio of the intersections of the immunostained cells per intersections of the whole islet. The total number of nuclei per islet, as well as the nuclei of the immunostained cells, were also tabulated. The percent number of each type of endocrine cell was calculated as the ratio of the immunostained nuclei to those of the entire islet. The absolute number of insulin, glucagon, somatostatin and pancreatic polypeptide cells per islet was also obtained via nuclear quantitation. These data were utilized to calculate the mean absolute number of each type of endocrine cell per islet. Percent volume, percent number and the mean absolute number of each type of endocrine cell were derived from the first 20 islets per pancreas. Islets were analyzed only if they contained at least 8 nuclei and were devoid of artifacts. As previously described, islets which were deranged by acinar cell displacement in KKAy mice, were not evaluated. All morphometry of the immunostained islets was done by an individual who was not familiar with animal identity.

Study 2

Assay of pancreatic islet hormone content. Immediately after termination, the splenic pancreas of KKAy ($n=6$) and C57BL/6 ($n=6$) mice was removed, weighed (wet weight) and frozen. Insulin, glucagon, somatostatin and pancreatic polypeptide were extracted from the pancreas by acid-alcohol. Radioimmunoassay of insulin and glucagon was described previously (Chang et al. 1977). Pancreatic somatostatin content was determined by the Somatostatin RIA Kit (Immuno Nuclear Co., Stillwater, MN, USA). Pancreatic polypeptide levels were measured by a double-antibody technique described previously (Gingerich et al. 1978).

Statistics. The metabolic, immunocytochemical, and hormone assay data were evaluated by the nonparametric Wilcoxon Rank-Sum Test. The pancreatic islet surface area data were analyzed by the ANOVA where the effects due to the islets were considered to be nested within the effects due to animals. Differences between KKAy and C57BL/6 mice were considered to be significant at $p<0.05$. All data are represented as the mean ± standard deviation.

Table 1. Terminal metabolic data of C57BL/6 and KKAy mice in studies 1 and 2

	C57BL/6	KKAy	*P* Value
Study 1			
Blood glucose (mg/dl)	136 ±17	334 ± 102	<0.001
Glycosylated hemoglobin (%)	2.4± 0.2	6.7± 1.3	<0.001
Plasma insulin (uU/ml)	64 ±60	2426 ±1475	<0.001
Plasma glucagon (pg/ml)	153 ±34	350 ± 104	<0.001
Study 2			
Blood glucose (mg/dl)	157 ±17	450 ± 80	<0.001
Glycosylated hemoglobin (%)	5.5± 0.3	14.6± 2.6	<0.001
Plasma insulin (uU/ml)	82 ±19	1620 ± 681	<0.001
Plasma glucagon (pg/ml)	258 ±90	355 ± 49	NS

NS: not significant

Results

Terminal metabolic data

Table 1 shows the terminal blood glucose, glycosylated hemoglobin, plasma insulin and plasma glucagon levels of the C57BL/6 and KKAy mice. In studies 1 and 2, the blood glucose, glycosylated hemoglobin and plasma insulin values were significantly elevated in KKAy compared to the C57BL/6 mice. The plasma glucagon values were significantly increased in KKAy mice in study 1. In study 2, the plasma glucagon levels showed a tendency to increase in the KKAy mice but the results were not statistically significant due to high values in the C57BL/6 mice.

Aldehyde-fuchsin stained islets

On the basis of the aldehyde-fuchsin stain, β-cells in the islets of C57BL/6 mice were heavily granulated (Fig. 1a) whereas those in the islets of KKAy mice displayed severe degranulation (Fig. 1b–d). The islets of C57BL/6 mice appeared unremarkable with respect to size and did not exhibit acinar cells within the endocrine tissue (Fig. 1a). Many of the islets of KKAy mice were hypertrophied (Fig. 1b, c) and displayed acinar cells among the endocrine tissue (Fig. 1c). In some cases, islets were difficult to identify in KKAy mice because of their degenerate condition characterized by a poorly defined capsule, exocrine cell displacement and paucity of endocrine cells (Fig. 1d). The presence of acinar cells within the endocrine tissue was observed in an average of 24% (range 2 to 55) of the examined islets in KKAy mice. It should be emphasized that exocrine cell displacement was not found in any of the examined islets of the C57BL/6 mice.

On the basis of natural numbers, the surface area of the pancreatic islets of KKAy mice appeared to be expanded in comparison with that of the C57BL/6 mice (mean ± standard deviation = 13867 $\mu m^2 \pm 15057$ for KKAy mice and 7053 $\mu m^2 \pm 7567$ for C57BL/6 mice). Due to excessive variability among the islet measurements in both groups of mice, a log transformation was conducted (mean ± standard deviation = 3.88 ± 0.06 for KKAy mice and 3.56 ± 0.06 for C57BL/6 mice). ANOVA analysis of the log transformed data (Fig. 2) revealed that the surface area of the islets in KKAy mice was significantly greater ($p < 0.001$) than that of the C57BL/6 mice.

Immunostained islets

The islets of C57BL/6 mice displayed a uniform microanatomy that consisted of a core of insulin cells (Fig. 3a) which were surrounded by a contiguous mantle of glucagon (Fig. 3b), somatostatin (Fig. 3c) and pancreatic polypeptide cells. The topographical arrangement of most islets in KKAy mice was markedly different from that of the nondiabetic mice. The insulin cells were distributed throughout most of the core and mantle of the islets (Fig. 4a). Some of the glucagon (Fig. 4b), somatostatin (Fig. 4c) and pancreatic polypeptide cells were displaced from the periphery to the core of the islets. The latter three types of cells appeared to lose continuity with one another and were often observed as isolated cells (Fig. 4b, c).

Table 2 shows the volume and numerical percents and mean absolute numbers of insulin, glucagon, somatostatin and pancreatic polypeptide cells in the pancreatic islets of C57BL/6 and KKAy mice. The volume and numerical percents and absolute number of insulin cells were significantly increased in islets of KKAy versus C57BL/6 mice.

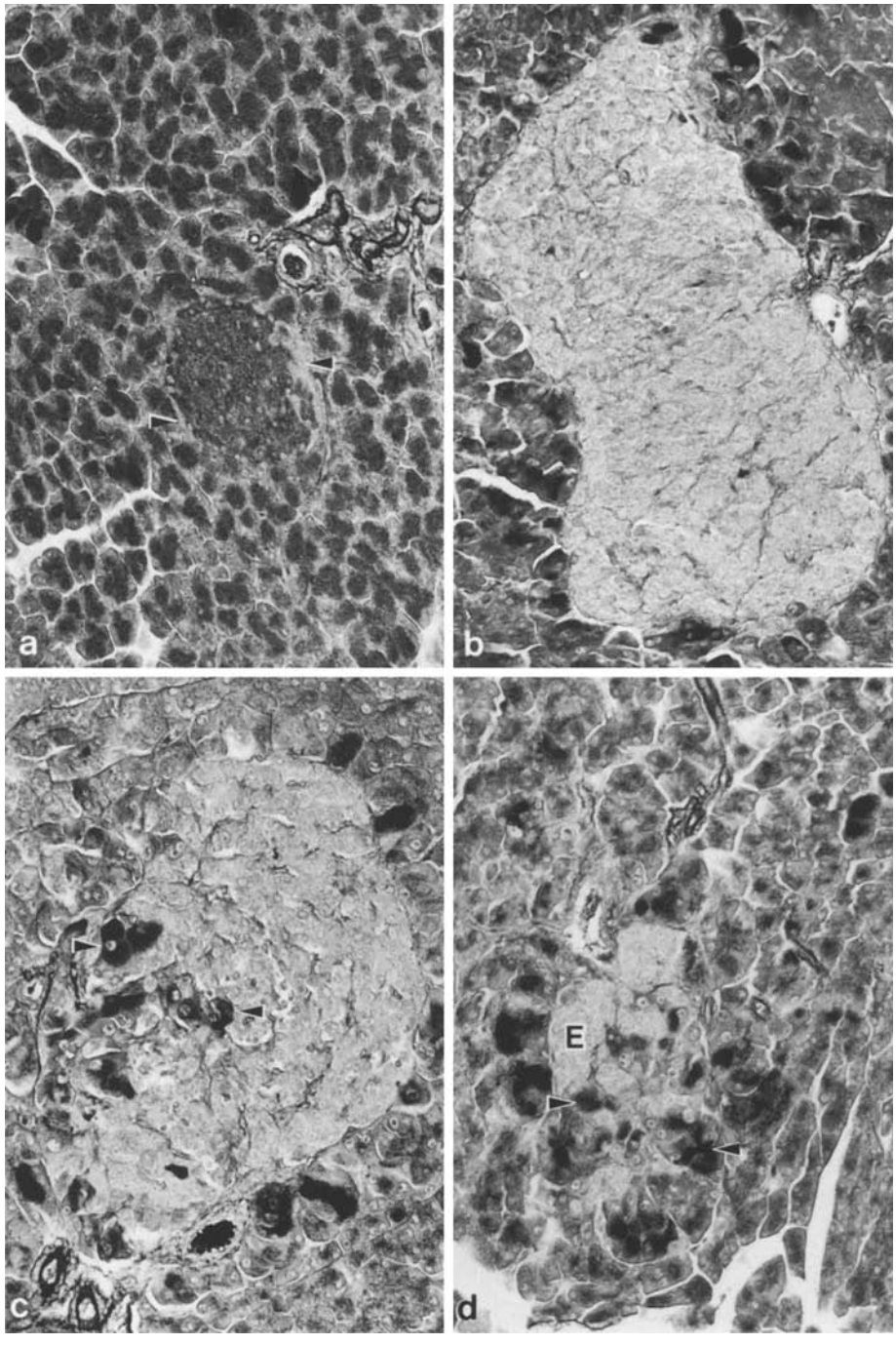

Fig. 1. **a** Pancreatic islet from a nondiabetic C57BL/6 mouse. Heavily granulated β-cells comprise the core of this islet. Note the peripheral unstained non-β-cells (*arrows*), unremarkable size, and lack of acinar cells in this islet. Aldehyde-fuchsin, approximately $\times 201$, **b** Pancreatic islet from a diabetic KKAy mouse. Severely degranulated β-cells are observable in this hypertrophied islet. Aldehyde-fuchsin, approximately $\times 201$, **c** Pancreatic islet from a diabetic KKAy mouse. Degranulated β-cells and some acinar cells (*arrows*) are detectable in this enlarged islet. Aldehyde-fuchsin, approximately $\times 201$, **d** Pancreatic islet from a diabetic KKAy mouse. The outline of this degenerate islet is difficult to determine because of severe acinar cell (*arrows*) displacement into the disorganized and sparse endocrine (*E*) tissue. Aldehyde-fuchsin, approximately $\times 201$

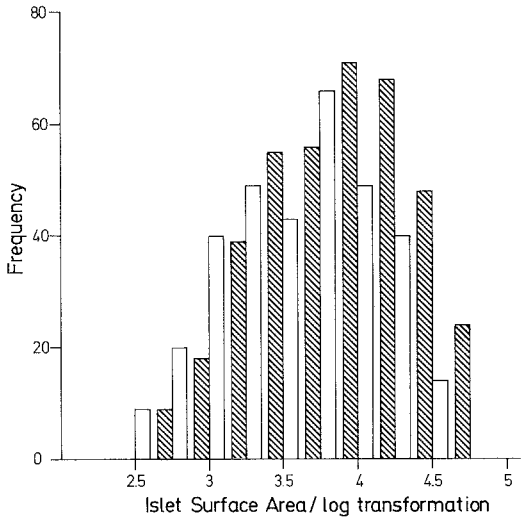

Fig. 2. Bar graph illustrating the expanded surface area (log transformed data) of the pancreatic islets of KKAy mice in comparison with that of C57BL/6 mice. ▨ diabetic; □ non diabetic

With respect to glucagon cells, the volume and numerical percents were significantly reduced in islets of KKAy mice whereas the absolute number was similar between the two groups of mice. The volume and numerical percents of somatostatin cells were significantly diminished in islets of KKAy mice. Although the absolute number of somatostatin cells was statistically similar between the two groups of mice, there was a tendency toward diminution in the islets of the diabetics. Morphometric analysis of pancreatic polypeptide cells did not reveal any statistically significant differences between the KKAy and C57BL/6 mice.

Pancreatic islet hormone content

Table 3 shows the pancreatic insulin, glucagon, somatostatin and pancreatic polypeptide contents in C57BL/6 and KKAy mice. The pancreatic insulin and somatostatin contents were significantly lower in KKAy compared to C57BL/6 mice. The pancreatic glucagon and pancreatic polypeptide levels did not differ between the two groups of mice.

Discussion

The results of the present study demonstrate that pronounced alterations were evident in the insulin cell population in the pancreatic islets of hyperinsulinemic, hyperglycemic KKAy compared with nondiabetic C57BL/6 mice. A concomitant increase in the volume and numerical percents of insulin cells in the KKAy mice imply that these cells are hyperplastic rather than hypertrophic. This contention is supported by the significant increase in absolute number of insulin cells in the KKAy mice. Although a slight increase in the volume percent of insulin cells has also been reported

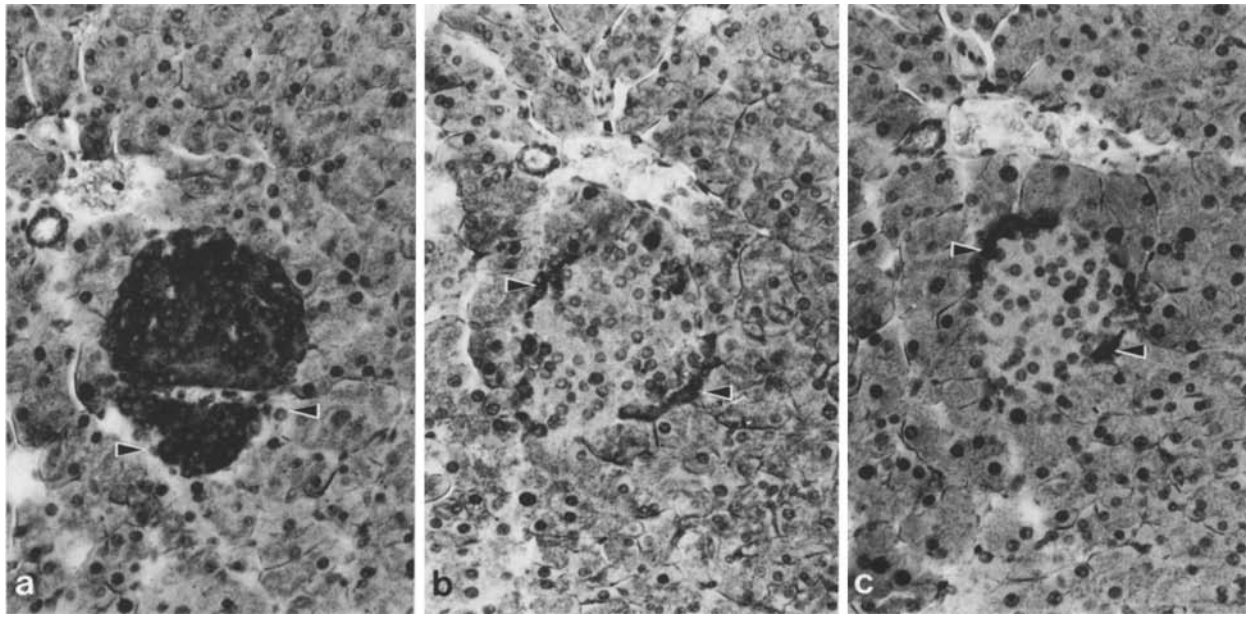

Fig. 3. a Immunostained (insulin) pancreatic islet from a nondiabetic C57BL/6 mouse. The core of this islet is composed of insulin cells which are surrounded by a contiguous mantle of unstained non-β-cells (*arrows*). PAP technique (insulin), approximately × 201, **b** Immunostained (glucagon) pancreatic islet from a nondiabetic C57BL/6 mouse. The glucagon cells (*arrows*) from a continuous layer along the periphery of the endocrine tissue. PAP technique (glucagon), approximately × 201, **c** Immunostained (somatostatin) pancreatic islet from a nondiabetic C57BL/6 mouse. The somatostatin cells (*arrows*) also are arranged in a continuous layer within the mantle of the endocrine tissue. PAP technique (somatostatin), approximately × 201

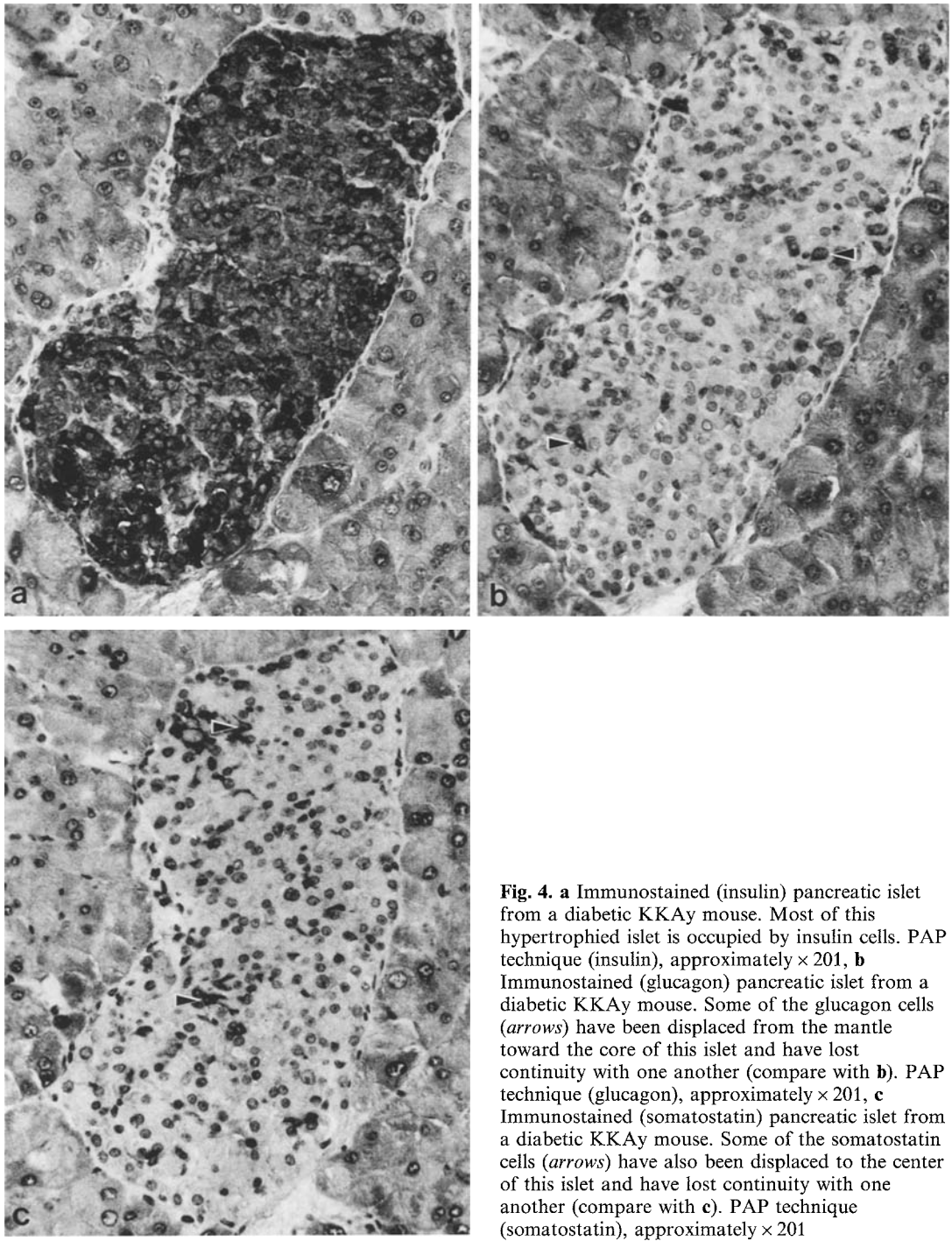

Fig. 4. a Immunostained (insulin) pancreatic islet from a diabetic KKAy mouse. Most of this hypertrophied islet is occupied by insulin cells. PAP technique (insulin), approximately × 201, **b** Immunostained (glucagon) pancreatic islet from a diabetic KKAy mouse. Some of the glucagon cells (*arrows*) have been displaced from the mantle toward the core of this islet and have lost continuity with one another (compare with **b**). PAP technique (glucagon), approximately × 201, **c** Immunostained (somatostatin) pancreatic islet from a diabetic KKAy mouse. Some of the somatostatin cells (*arrows*) have also been displaced to the center of this islet and have lost continuity with one another (compare with **c**). PAP technique (somatostatin), approximately × 201

for the islets of ob/ob mice on the C57BL/6J background, the results were not statistically significant (Baetens et al. 1978). This difference in degree of increase between the ob/ob and KKAy mice may be related to the fact that the KKAy mice are younger and display a more severe diabetes characterized by higher blood glucose levels. It is also possible that dissimilar genetic composition of the KKAy and ob/ob mice may promote different influences on insulin cell proliferation. The metabolic data of this investigation and earlier studies (Iwatsuka et al. 1970; Chang et al. 1986) revealed that KKAy mice exhibit augmented plasma insulin levels despite the fact that approximately 25% of the islets were degenerate. Furthermore, since the insulin cells were markedly degranulated and the pan-

Table 2. Volume and numerical percent and mean absolute number of insulin, glucagon, somatostatin and pancreatic polypeptide cells in the pancreatic islets of C57BL/6 and KKAy mice

	C57BL/6	KKAy	*P* Value
Insulin Cells			
Volume %	74 ± 2	84 ± 3	<0.001
Numerical %	75 ± 3	84 ± 2	<0.001
Absolute Number	57 ±13	121 ±25	<0.001
Glucagon Cells			
Volume %	12 ± 4	6 ± 2	<0.002
Numerical %	12 ± 4	7 ± 3	<0.003
Absolute Number	9 ± 3	9 ± 5	NS
Somatostatin Cells			
Volume %	11 ± 5	4 ± 3	<0.001
Numerical %	11 ± 4	5 ± 6	<0.005
Absolute Number	9 ± 4	6 ± 3	NS
Pancreatic Polypeptide Cells			
Volume %	1.3± 0.9	0.7± 0.4	NS
Numerical %	1.6± 0.9	1.0± 0.4	NS
Absolute Number	2 ± 1	1 ± 1	NS

NS: not significant

Table 3. Pancreatic insulin, glucagon, somatostatin and pancreatic polypeptide content in C57BL/6 and KKAy mice

	C57BL/6	KKAy	*P* Value
Insulin (U/g)	4.84 ± 0.63	3.06 ± 1.10	<0.009
Glucagon (μg/g)	4.961± 1.575	4.406± 1.012	NS
Somatostatin (ng/mg)	0.598± 0.188	0.174± 0.062	<0.002
Pancreatic Polypeptide (ng/g)	267.6 ±59.7	215.3 ±53.3	NS

NS: not significant

creatic insulin content was depressed, it seems that the insulin cell population was severely stressed to synthesize, store and secrete insulin in an attempt to compensate for insulin resistance (Iwatsuka et al. 1970) in the peripheral tissues. Future investigation will be necessary to determine if the insulin cell abnormalities precede or follow the insulin resistance in this animal model.

A decrease in volume and numerical percents of the glucagon and somatostatin cells was observed in the pancreatic islets of KKAy mice. However, the absolute numbers of these endocrine cells were unchanged in the diabetic compared to the nondiabetic mice. These data suggest that the absolute numbers of glucagon and somatostatin cells remain stable in the KKAy mouse up to 165 days of age despite massive proliferation of the insulin cells. Thus, it seems reasonable to conclude that the downward shift in the volume and numerical percents of the non-insulin cells and the enlargement of the islets in the KKAy mouse appear to be predominantly a manifestation of insulin cell multiplication. Morphometric analysis of pancreatic islets from ob/ob mice on the C57BL/6J background have also confirmed that the fractional volumes of glucagon and somatostatin cells are lower with respect to an expanding insulin cell population (Baetens et al. 1978).

The metabolic data of the current study infer that plasma glucagon was elevated in the KKAy mice. Since pancreatic glucagon content was similar in KKAy and C57BL/6 mice, it appears that the glucagon cells synthesized enough hormone to compensate for increased secretion. The etiology of hyperglucagonemia in the KKAy mouse is unclear. It may be related to the extremely low level of pancreatic somatostatin which, in normal amounts, is known to inhibit insulin and glucagon release (Efendic et al. 1974; Fijimoto et al. 1974). High levels of plasma insulin and glucagon accompanied by low pancreatic somatostatin content have also been reported in the ob/ob mouse (Bae-

tens et al. 1978). It was proposed that hyperinsulinemia in the ob/ob mouse may supress somatostatin secretion from the pancreatic islets by a negative feedback mechanism or that a reciprocal relationship between circulating insulin and pancreatic somatostatin may exist (Patel et al. 1977). If the second hypothesis is correct, it would be important to determine if the earliest defect in the KKAy mouse involves deranged insulin or somatostatin cell secretion.

The topographical arrangement of the endocrine cells within the pancreatic islets of the KKAy mice was markedly disrupted in comparison with those of C57BL/6 mice. Many of the glucagon, somatostatin and pancreatic polypeptide cells were displaced from the mantle to the core of the islet, probably by expansion of the insulin cell population. Furthermore, the non-insulin cells seemed to lose contact with one another and assume a more isolated position within the core of the islet. Previous light microscopic analysis has shown comparable abnormalities in the distribution of the non-insulin cell populations of the ob/ob mouse (Baetens et al. 1978). It was also hypothesized that topographical disturbances in the pancreatic islets of the ob/ob mouse may be detrimental to endocrine cell communication and have deleterious effects on hormone secretion (Baetens et al. 1978). Thus, it seems feasible that hypersecretion of insulin in the KKAy mouse may be further exacerbated by the displacement and isolation of somatostatin cells which are known to inhibit insulin and glucagon secretion (Efendic et al. 1974; Fijimoto et al. 1974). Although the incidence varied considerably among the KKAy mice, derangement of islet integrity was further compromised by acinar cells among the endocrine cells. The presence of exocrine cells in the islet tissue has also been described in db/db mice on the C57BL/6KsJ background (Coleman and Hummel 1967; Coleman 1978) and considered to be early evidence of degeneration. Since the KKAy mice in the present study displayed extremely high levels of plasma insulin, it is obvious that a sufficient quantity of viable islets were able to maintain an excessive output of insulin. It would be interesting to determine if the islets of older KKAy mice display a greater incidence of atrophy which may ultimately lead to severely reduced levels of plasma insulin as is the case with the db/db mouse (Coleman 1978).

In conclusion, this study has demonstrated that the hypertrophic pancreatic islets of KKAy mice are a manifestation of insulin cell hyperplasia which appears to elicit alterations in the percent number and volume and anatomical location of the non-insulin cells. The quantitative and qualitative defects in the endocrine cells may, in turn, be the cause or consequence of deranged hormone synthesis, storage and/or secretion in the islets of the KKAy mouse. Future studies will focus on temporal elucidation of the functional defects in the pancreatic islets and peripheral insulin receptors of this animal model.

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