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Insulin and C-peptide co-localization in the β granules of normal human pancreas and insulinomas

A quantitative immunocytochemical approach

Moshe Kalina[1], Lars Grimelius[2], Bjorn Cedermark[3], and Ilan Hammel[4]

[1] Department of Histology and Cell Biology, Sackler School of Medicine, Tel Aviv, Israel, 69978
[2] Department of Pathology, Medical School, Uppsala University, S-75185, Uppsala, Sweden
[3] Surgical Unit, Carolinska Hospital, Stockholm, Sweden
[4] Department of Pathology, Sackler School of Medicine, Tel Aviv, Israel, 69978

Summary. It has been shown, by using the immunogold technique, that C-peptide and insulin are co-localized in the mature granules of human pancreatic β cells and insulinomas with typical granules. The mean gold bead densities of both C-peptide and insulin were at least twice as high in the normal pancreas when compared with the insulinomas. The mean granule diameter of the insulinoma cells ($D = 0.30 \pm 0.12$ μm) was smaller than that of human pancreatic cells ($D = 0.45 \pm 0.15$ μm). The morphometric data indicate that each of the antigens (C-peptide and insulin) is distributed similarly in the halos and the dense cores of the β granules. Thus, no topological segregation of these two antigens occurs within the β granules of either normal human pancreas or insulinomas.

Key words: C-peptide – Insulin – Pancreatic β cells – Insulinoma morphometry – Immunocytochemistry

Introduction

It has been shown by using the colloidal gold technique, that C-peptide and insulin are co-localized in the mature granules of the pancreatic β cells (Orci 1982; Orci 1985; Hammel and Kalina 1989). These peptides have also been demonstrated in non-osmicated tissue from human insulin producing tumours (Varndell et al. 1982). Recently Berger et al. (1988) used the immunogold technique to demonstrate insulin in the β granules of various types of human pancreatic insulinomas with typical and atypical granules.

The main advantage of the immunogold technique is the relative ease with which quantitation may be done by morphometric analysis in correlation with well-preserved ultrastructure in osmicated tissues (Kalina et al. 1988; Hammel and Kalina 1989). In a previous study we used the immunogold technique for investigating the correlation between the expression of antigenic sites and size of granules in β cells of rat pancreas (Hammel and Kalina 1989). It was demonstrated that the distribution patterns of antigenic sites for C-peptide (which also represents proinsulin sites) and insulin are similar when granule size distribution is correlated with the density of antigenic sites.

In the present communication we describe a quantitative study of the distribution of C-peptide and insulin in the secretory granules of human pancreatic β cells compared to their distribution in human insulin-producing tumours (insulinomas). An attempt was also made to study the relative distribution of both antigens (C-peptide and insulin) within the β granules of normal human pancreas and insulinomas. Our quantitative data indicate that each antigen is distributed similarly in the halos and the dense cores of the β granules. Thus, no topological segregation of these antigens occurs within the β granules.

Materials and methods

Normal human pancreas was obtained during surgery. Insulinoma tissue was obtained from two tumours, clinically diagnosed as benign functional insulinomas. Morphologically they were classified as tumours storing only tpyical beta granules (type I) (Creutzfeldt et al. 1973). Both tissues were immunoreactive with anti insulin and anti C-peptide antibodies at the light microscopical level. No tumour immunoreactivity was found using antisera directed against glucagon, somatostatin, gastrin, pancreatic polypeptide (PP) or vasoactive intestinal peptide (VIP). Tissue blocks (1 mm^3) were fixed for 3 h in 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature. After fixation, tissues were postfixed in 1% osmium tetraoxide (in

Offprint requests to: M. Kalina

cacodylate buffer), dehydrated in graded series of alcohols, and embedded in araldite. Thin sections (0.075±0.015 μm) were collected on uncoated nickel grids.

Anti-insulin (raised in guinea pigs) was obtained from Bio-Yeda (Rehovot, Israel) and used in dilution of 1/1000. Rabbit anti C-peptide was obtained from EY laboratories (Ca) and was used in the same dilution. The immunocytochemical procedures were as previously described (Hammel and Kalina 1989). Briefly, sections were treated with a saturated aqueous solution of Na-metaperiodate for 30 min at room temperature to reduce the protein masking effect of osmium (Bendayan and Zollinger 1983). The sections were then washed and placed in normal goat serum (1:20 dilution) for 30 min. After draining, sections were exposed to the primary antisera for 18 h at 4° C. After rinsing, the sections were incubated (1 h at room temperature) in goat anti rabbit IgG-gold (10 nm) for C-peptide and goat anti guinea pig IgG-gold (10 nm) for insulin. Both reagents (Jansen Pharmaceutica, Beerns, Belgium) were diluted 1:15. The grids were counterstained with uranyl acetate and lead citrate. Specimens were viewed in JEM 100B electron microscope.

Specificity of the immunolabelling was assessed by incubating the sections with non-immune sera. No labeling beyond the background was obtained in the experimental system. It was previously found that no cross-reactivity existed between the anti C-peptide and insulin. However, cross reactivity was observed between anti C-peptide and proinsulin (Hammel and Kalina 1989).

All morphometric measurements were performed using transmission electron micrographs (×45000; original magnification was ×20000) as explained elsewhere in detail (Hammel et al. 1987; Kalina et al. 1988). Briefly, the cross sectional area (a_i) of each individual (i-) granule was measured, using a graphic digitizing tablet (Hewlett-Packard 9111A, USA) interfaced to a desk top technical micro-computer (HP 9817H). The resulting processed data were plotted using an HP 9872C graphics plotter.

Gold bead labeling density in the various compartments was determined by using the following procedures. The area (a_i) of each granule was measured. The number (N_i) of gold beads of each individual granule was recorded and the granule gold bead density was calculated (Bendayan 1984; Kalina et al. 1988).

$$\text{gold bead density} = \sum_{i=1}^{i=n} N_i \Big/ \sum_{i=1}^{i=n} a_i. \quad (1)$$

To determine gold bead distances from the granule membrane we have used those granules in which both electron dense cores and membranes were well defined. The shortest distance between the gold beads and the membranes was measured using the digitizer. The calculated gold bead densities and distances in the granules were converted to cumulative frequency histograms. Statistical comparison analysis between the cumulative curves was performed by using the Kolmogorov-Smirnov Test or the x^2-test (Sokal and Rohlf 1969).

Results

A typical distribution of gold immunolabelling of both C-peptide and insulin in a well granulated human insulinoma with typical β granules (type I according to Creutzfeldt et al. 1973) is represented in Fig. 1. Gold labelling of both C-peptide and in-

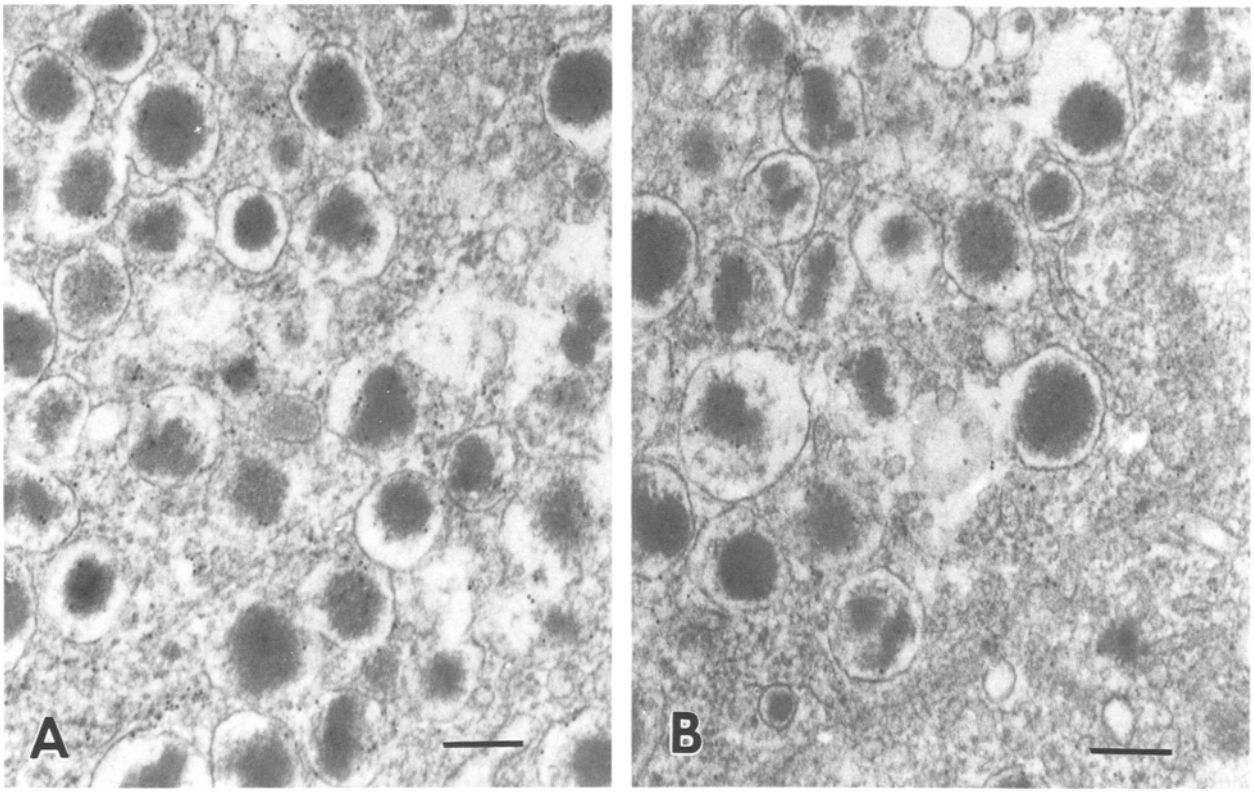

Fig. 1a, b. Portion of β cell from human insulinoma tumour immulabeled for C-peptide (**a**) and insulin (**b**) using 10 nm gold beads. ×50000. *Bar*-1 μm

Table 1. Density of gold beads in the mature β granules of β cells of normal pancreas and insulinomas[a]

		C-peptide gold bead density	Insulin (gold beads/ μm^2)
Human Pancreas (normal)	Exp. 1	91 ± 20 ($N=243$)	124 ± 21 ($N=256$)
($D=0.45 \pm 0.15$ μm)[b]	Exp. 2	–	71 ± 11 ($N=282$)
Insulinoma	Case 1	38 ± 2 ($N=458$)	34 ± 2 ($N=705$)
($D=0.30 \pm 0.12$ μm)[b]	Case 2	–	33 ± 3 ($N=241$)

[a] Quantitative evaluation of the number of gold beads over mature β-granules. The data obtained from 25 cells (for each normal pancreas) and 50 cells (for each case of insulinoma). The numbers in parentheses indicate the number of granules measured

[b] D represents the mean diameter of mature secretory granules in human pancreas and insulinoma

sulin was found mainly, but not exclusively, in the dense core. Some gold particles were found also over the halos. Similar distribution of gold immunolabelling for both C-peptide and insulin was observed in normal human pancreas. The gold beads were distributed over the whole granule, i.e. in the halo as well as in the crystalloid or amorphous dense core of secretory granules. Table 1 summarizes the quantitative data of C-peptide and insulin gold labelling in both normal β cells and insulinomas. The labelling density (gold beads/μm^2) of both C-peptide and insulin were similar in each of the tissues (normal pancreas and two insulinomas). However, labelling densities of the insulinomas β-cells were 3–4 times lower than those of normal pancreatic cells.

An attempt was made to quantify the relative distribution of C-peptide and insulin gold immunollabel over the halos and dense cores of the β-granules. This was done by measuring the distances of the gold particles and the dense cores from the β granules' limiting membranes. Histograms of threse distances are presented in Fig. 3. The immunogold labelling of both C-peptide and insulin in human pancreas is distributed similarly in both the halo and the dense core. (Approximately 50% of the gold particles are located over the halo and 50% over the dense core). In contrast with the normal human pancreas, the immunogold labelling of C-peptide and insulin in the insulinomas is distributed mostly (over 70%) in the dense core.

Discussion

The immunohistochemical distribution of C-peptide and insulin, as expressed by the immunogold

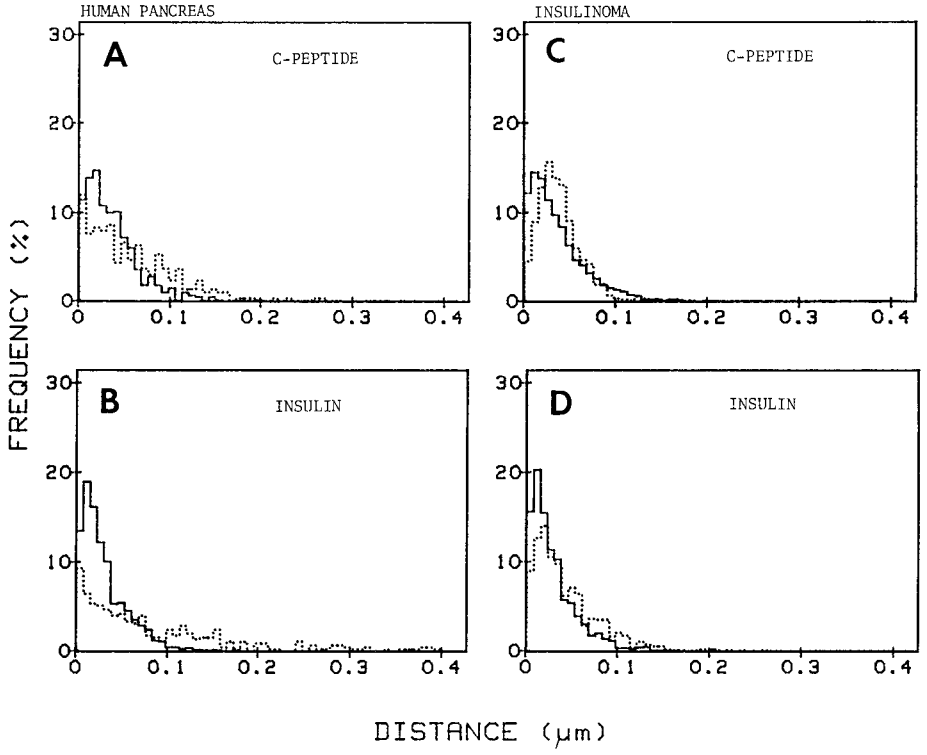

Fig. 2A–D. Frequency histograms represent gold beads distances from the secretory granules membrane (...). The distances of the dense core edge from the granule membrane is represented by the smooth line (___). Histograms **A** and **B** represent the labeling of C-peptide and insulin in normal human pancreas; histograms **C** and **D** the labeling of C-peptide and insulin in insulinoma

technique, is presented in the current work. A comparison is made between normal pancreas and insulin producing tumours (insolinomas) with regard to the cellular distribution and topological segregation of these two peptides in β granules. The type I insulinomas of the present study were tumours consisting of cells storing only typical β granules (classification according to Creutzfeldt et al. 1973). Berger et al. (1988) demonstrated, by the immunogold technique, the presence of insulin in the β granules of this tumour.

Mean gold bead densities of both C-peptides and insulin were at least twice as high in the normal pancreas when compared with the insulinomas (Table 1). The mean granule diameter of the granules of insulinoma cells ($D = 0.30 \pm 0.12$ µm) was smaller than that of human pancreas ($D = 0.45 \pm 0.15$ µm). These two factors may explain the weak light microscope staining that is often obtained when immunostaining insulinomas (even type I with typical β granules) with antibodies to insulin or C-peptide, compared with normal pancreatic islets. The ratio of mean gold bead density for insulin to C-peptide in the mature β granules of insulinomas was approximately 1:1. This ratio reflects the conversion reaction of proinsulin to insulin (Fletcher et al. 1981; Steiner 1976). A slightly higher ratio was observed in β granules of human pancreas. These results are similar to those observed in rat pancreas (Hammel and Kalina 1989) where it was found that 45% of the granules had the ratio insulin: C-peptide of 1:1; the other granules had a higher ratio. Since the gold bead density in each granule profile is a function of many factors, significant fluctuations in the ratio may be expected (For detailed analysis see Hammel and Kalina 1989).

There is evidence to support the notion that proinsulin conversion to insulin is a post Golgi event that probably occurs in the immature clathrin-coated granules (Orci 1984; Oric et al. 1985; Hammel and Kalina 1989). Such an in-granule processing of proinsulin may result in topologically segregated C-peptide and insulin. A possible topological selective distribution of C-peptide and insulin between the halos and dense cores of the β granules has been debated for a long time. Steiner et al. (1975) suggested that the halos stored the C-peptide, whereas insulin was packaged in the dense core. Orci (1982) showed in dog pancreas that sites immunoreactive to insulin are selectively present over the core of the β-granule, whereas C-peptide was demonstrated over the clear halos but not on the cores. Our previous data did not support such a topological segregation in rat β-cells (Hammel and Kalina 1989) where both C-peptide and insulin were mainly located in the dense core. By using a quantitative approach we were able to demonstrate, in the present communication, that both peptides were similarly distributed in the halos and dense cores of the β granules in both normal human pancreas and insulinomas. In the normal pancreas the labelling of both peptides was evenly distributed between the dense core and the halo, while, in the insulinoma β granules, most of the label for both peptides was concentrated over the dense core (over 70%). However, in both tissues, no selective distribution of the two peptides was observed between the halo and dense core. These results are similar to those described by Varndell et al. (1982) who found that in non-osmicated insulinoma both peptides are mainly localized in the dense core. Berger et al. (1988) also found in type I insulinoma that insulin is mainly located in the dense core. These results differ from those observed for the distribution of glycentin and glucagon in the A cells of the pancreatic islets (Ravazzola and Orci 1980; Varndell et al. 1982).

Glucagon and glycentin-like material are topologically segregated in the α granule of the pancreatic A cell. The mantle (periphery) of the α granules which is labeled with glycentin is also stained with the Grimelius silver technique (Bussolati et al. 1971) as well as with chromogranin A (Ehrhart et al. 1986).

The variability in the distribution of C-peptide and insulin immunoreactive sites (visualized by gold beads) between the core and halo of the β granules may partly be attributed to technical reasons. It has been shown that variation in gold labelling of β cells is due to the use of different fixation and embedding procedures (Orci et al. 1984). Similar differences in gold labelling were observed in other tissues fixed and embedded according to various protocols (Abramson 1986). Therefore, any immunocytochemical comparative study (quantitative as well as qualitative) must be performed with tissues fixed and processed in a similar manner. Both normal human pancreas and insulinoma were processed similarly. When these two tissues were processed in Lowicril k4M and labeled with C-peptide and insulin, higher gold densities than those described were found (unpublished data). However, the differences in gold densities between the two tissues, as well as the intragranular localization were similar to that described in the present communication. We may conclude that it is reasonable to assume that no topological segregation occurs in the β granules and the conflicting observations made by various investigators are merely due

to variations in the fixation and processing protocols of the tissues studied.

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References

Abramson DR (1986) Post embedding colloidal gold immunolocalization of laminin to the lamina rara interna, lamina densa, and lamina rara externa of renal glomerular basement membranes. J Histochem Cytochem 34:847–853

Bendayan M, Zollinger M (1983) Ultrastructural localization of antigenic sites on osmium-fixed tissues applying the protein A gold technique. J Histochem Cytochem 31:101–109

Berger G, Berger F, Dutrieux N, Guilland MH, Vauzell J (1988) Electron microscope localization of insulin-like immunoreactivity of conventionally processed human insulinomas. Virchows Arch [A] 412:443–450

Bussolati G, Capella C, Vassalo G, Solcia E (1971) Chemical and ultrastructural studies on pancreatic A cells. Evidence for glucagonand non-glucagon components of the granule. Diabetologia 7:181–188

Creutzfeldt W, Arnold R, Creutzfeldt C, Deuticke U, Frerichs H, Tracks NS (1973) Biochemical and morphological investigation of 30 insulinomas: correlation between the tumor content on insulin and proinsulin-like components and the histological and ultrastructural appearance. Diabetologia 9:217–231

Ehrhart M, Graube D, Bader MF, Aunis D, Gratzl M (1986) Chromogranin A in the pancreatic iselt: cellular and subcellular distribution. J Histochem Cytochem 34:1673–1682

Fletcher DJ, Quigley JP, Bauer GE, Noe BD (1981) Characterization of proinsulin- and proglucagon-converting activities in isolated islet secretory granules. J Cell Biol 90:312–322

Hammel I, Dvorak AM, Galli SJ (1987) Defective cytoplasmic granule formation I. Abnormalities affecting tissue mast cells and pancreatic acinar cells of Beige mice. Lab Invest 56:321–328

Hammel I, Kalina M (1989) Insulin and C-peptide (Proinsulin) packaging: A quantitative immunocytochemical approach. J Elect Micro Tech 10:1–7

Kalina M, Elmalek M, Hammel I (1988) Intragranular processing of pro-opiomelanocortin in the intermediate cells of the rat pituitry glands. Histochemistry 89:193–198

Orci L (1982) Macro- and micro-domains in the endocrine pancreas. Diabetes 31:538–565

Orci L, Ravazola M, Amherdt M, Yanaihara C, Yanaihara N, Halban P, Renold AE, Perrelet A (1984) Insulin, not C-peptide (proinsulin) is present in crinophagic bodies of the pancreatic β cell. J Cell Biol 98:222–228

Orci L (1985) The insulin factory: Assembly line. Diabetologia 28:528–546

Orci L, Ravazola M, Amherdt M, Madsen O, Vassalli J-D, Perrelet A (1985) Direct identification of prohormone conversion site to insulin-secreting cells. Cell 42:671–681

Ravazola M, Orci L (1980) Glucagon and glicentin immunoreactivity are topologically segregated in the granule of the human pancreatic A cell. Nature 284:66–67

Steiner DF, Kemmler W, Tager HS, Peterson Jo (1974) Proteolytic processing in the biosynthesis of insulin and other proteins. Fed Proc 33:2105–2115

Varndell IM, Tapia FJ, Probert L, Buchan AMJ, Gu J, De Mey J, Bloom SR, Polak JM (1982) Immunogold staining procedure for the localisation of regulatory peptides. Peptides 3:259–272