Relevant infrastructural alterations in a pancreatic neuroendocrine tumor: an insulinoma case

GABRIEL-VALERIU MIRANCEA¹, ANA-MARIA MOROŞANU², SIMONA CARNICIU³, SIMONA DIMA⁴, NICOLAE BACALBAŞA¹, IRINEL POPESCU¹, CONSTANTIN IONESCU-TÎRGOVIŞTE⁵, NICOLAE MIRANCEA²

¹) “Carol Davila” University of Medicine and Pharmacy, Bucharest, Romania
²) Institute of Biology Bucharest of Romanian Academy
³) “Nicolae C. Paulescu” National Institute of Diabetes, Nutrition and Metabolic Diseases, Bucharest, Romania
⁴) “Fundeni” Clinical Institute, Bucharest, Romania

Abstract
In this study, we focus our interest on some peculiar infrastructural abnormalities detected in an insulinoma case. Tumor pancreatic endocrine cells proliferated detrimental to exocrine counterpart, so that extensive areas of prevalent β-tumor cells can be seen. Two phenotypes of β-tumor cells can be identified: (1) β-tumor cells with full euchromatic and nucleolated nuclei and (2) β-tumor cells with heterochromatic and shrink nuclei. Because of stroma alteration, including basement membrane, cell–extracellular matrix junctions are also compromised. The mostly striking and important finding in this report for a case of insulinoma is the high fragility of plasma membrane of both two phenotypes of β-tumor cells. Cell–cell junctions, especially desmosomal junctions are severely altered, almost missing, plasma membranes showed shedding membrane vesicles and extensive dissolutions leading to pseudo-syncytia formation. Extravasated blood cells, including inflammatory cells contribute to the dramatic and extensive destructive areas of epithelial cells as well as stroma counterpart. Moreover, also the inner cell cytomembranes exhibit abnormalities: many β-tumor cells have excessive dilatations of nuclear envelope and endoplasmic reticulum. All above severe infrastructural abnormalities, especially down regulation of cell–cell and cell–extracellular matrix adhesions and plasma membranes fragility might result in aberrant cell behavior and, consequently, much care should be taken for the postoperatory patient evolution.

Keywords: insulinoma, β-tumor cells, plasma membrane fragility, pseudo-syncytia.

Introduction
According to the American Cancer Society, nowadays, pancreatic cancer is considered as the fourth leading cause of cancer death, with the majority occurring in people 60 years of age or older in western countries. Mostly of pancreatic malignant tumors involve exocrine pancreas. Endocrine component of the pancreas can be also involved in developing tumors, so-called pancreatic neuroendocrine tumors (PNETs), but mention must be made that almost are benign tumors. Less than 3% of primary pancreatic neoplasms result from neuroendocrine tumors [1]. PNETs are a sub-group of gastroenterohepatic neuroendocrine tumors (GEP-NETs). There is a high heterogeneity of cell types involved in GEP-NETs (endocrine cells that express and share a number of antigens with nerve elements, like neuron-specific enolase [2, 3]. In this context, mention must be made that Treutelaar et al. [4] reported that both in vivo and experimental model pancreatic endocrine cells arise independently of nestin-positive precursors, nestin-positive cells playing an important role in the growth and maintenance of the islet. Usually, the involved cells showed hormone overproduction and oversecretion. GEP-NETs are classified as “functional” (F-NETs) or non-functional (NF-NETs). GEP-NETs may have (1) benign, (2) uncertain or (3) malignant behavior. To date, at least 15 different cell types of neuroendocrine system of the gut have been described [5].

There are two important clinical characteristics to discriminate for the type of pancreatic Langerhans islet cell tumors: (1) the predominant type of hormone they secrete and (2) their ability to metastasize.

Normal human endocrine pancreas is represented by more than one million of small islet cells. In non-neoplastic tissue α, β, δ, ε and PP (F) cell types of pancreatic islets may be identified electron microscopically (including immune electron microscopy) by their specific granules. Each above five-cell type of endocrine pancreas secretes specific hormone α-cells (synthesizing glucagon hormone), β-cells (insulin, amylin and C-peptide), δ-cells (somatostatin), PP-cells (pancreatic polypeptide) and ε-cells (ghrelin) involved in different body function [6].

Endocrine component of the pancreas represents less than 2% of the whole pancreas volume, but it plays a crucial role in regulating metabolic functions, mainly the glucose metabolism. The concentration of glucose in blood stream is a very important parameter of human body homeostasis. In abnormal conditions, dysfunction of β-cells leads to two major diseases (type I or type II of diabetes) and insulinoma. Pancreatic islet neoplasms are rare endocrine tumors. The most common type is of β-cell origin known as insulinoma, which can be (1) either benign or (2) malignant [7]. An insulinoma is a tumor, usually benign (non-cancerous), made up of specialized β-pancreatic islet cells that are able to constantly secret insulin, causing hypoglycemia, known as hyperinsulinemic
hypoglycemia (paroxysmal hypoglycemic crisis). It is well known that the normal function of the brain requires constantly supply with high amounts of oxygen and glucose, a glucose level below 50 mg/dL resulting in abnormal, dangerous brain-related symptoms (so-called neuroglycopenia). Patients with insulinoma present Whipple’s triad: (1) hypoglycemic symptoms described as either autonomic or neuroglycopenic/neuropsychiatric disturbance, neuromuscular signs, (2) low-plasma glucose level (less than 50 mg/dL at the time of hypoglycemic symptoms), and (3) immediate relief of the above-mentioned symptoms with administration of exogenous (oral or intravenous) glucose.

Hyperinsulinemia and consequently, hypoglycemia are hallmarks of insulinoma. Excessive or inappropriate secretion of insulin and proinsulin leads to hypoglycemia – one of the first symptoms of insulinoma. Moreover, a patient developing an insulinoma experiences visual disturbances, palpitations, sweating, difficulty speaking, confusion and abnormal behavior. Up to half of patients suffering of insulinoma experience episodic unconsciousness, 10–15% will suffer seizures and 20% exhibit weight gain. Normal individuals from the human population have a 6:1 ratio of insulin/proinsulin, whereas patients affected by insulinoma exhibit a severe imbalance of insulin/proinsulin ratio, closer to 1:1 [7].

Statistical data indicate that insulinoma is a rare disease: it counts for 1–5 cases per million persons/year [7, 8]. Different from the other islet cell tumors such as gastrinoma (Zollinger–Ellison syndrome) and VIPoma, most insulinomas are considered to be benign tumors. Approxi-mately 90% from insulinomas are benign adenomas and only 10% are malignant tumors giving metastasis in lymph nodes, liver and rarely in bones. The presence of metastasis is the only clinical sign to consider an insulinoma as malignant tumor [8]. Benign insulinoma tumors are solitary and less than 2–5 cm in diameter when detected. Insulinomas that turn out to be malignant usually have a diameter over 3 cm, and about 1/3 have metastasizing at the time of diagnosis.

So far, there is no specific morphologic, biochemical or genetic trait to discriminate between benign and malignant tumors in case of islet cell pancreatic tumors. Malignancy is usually associated with metastasis. Of course, before to metastasize, some specific cell behavior takes place in order to perform invasion of endocrine pancreatic malignant transformed cells. In this context, we may assume that transmission electron microscopic investigations may provide relevant information about putative malignant insulinoma development. Here, we investigate by electron microscopy a case of insulinoma. We underline that, to some extent, some infrastructural abnormalities can be considered to evaluate the benign and malignant insulinoma.

Materials and Methods

We report a case of insulinoma in a 37-year-old male patient. At the time of referral to the “Dan Setlacec” Center of General Surgery and Liver Transplantation of “Fundeni” Clinical Hospital, Bucharest, Romania, he has recurrent episodes of hypoglycemia.

The abdominal contrast enhanced tomography (CT scan) confirmed the presence of the 1 cm tumoral lesion in the body of the pancreas (Figure 1). The patient underwent distal pancreatectomy with spleen preservation and intraoperative ultrasound.

Figure 1 – Preoperative computed tomography showing a pancreatic tumor (arrow).

The pancreatic samples were classified based on revised WHO (version 2010, neuroendocrine tumors grade 1 or NET G1, neuroendocrine tumors grade 2 or NET G2 neuroendocrine carcinomas or NEC of grade G3. Tumor staging was carried out using ENET–TNM (European Neuroendocrine Tumor Society). For grading, the Ki67 index was assessed using the MIB1 clone antibody (Dako, Glostrup, Denmark), as the percentage of Ki67 positive cells among 2000 tumor cells in areas where the highest nuclear labeling was noticed.

In order to perform transmission electron microscopy (TEM) investigations, small tissue fragments about 2–3 mm3 from a normal pancreas, from the pancreatic tumor mass as well as from the peri-pancreatic tumor but uninvolved part of the pancreas (as control counterpart tissue) resulted by surgery as curative therapy for the patient suffering from presumptive insulinoma (surgeon got patients’ consent) were processed following the routine TEM protocol [9]. After pre-fixation in fresh ice-cold 4% glutaraldehyde in sodium cacodylate buffer, pH 7.4 for three hours at 4°C, the tissues were six times washed in 0.05 M sodium cacodylate buffer, pH 7.4 at 4°C, the tissues were six times washed in 0.05 M sodium cacodylate buffer (pH 7.4) at 4°C, post-fixed in 2% osmium tetroxide in 0.1 M sodium cacodylate at 4°C for 2.5 hours, stained en bloc with 0.5% aqueous uranyl acetate overnight at 4°C and washed with 0.05 M sodium cacodylate buffer, pH 7.4. After dehydration in graded series of ethanol and infiltration with propylene oxide, specimens were embedded in Glycid ether (Epon 812-equivalent) and finally polymerized at 60°C for 48 hours. Semithin sections were stained with 1% toluidine blue for light microscopy. Ultrathin sections (80–100 nm) were cut using a diamond knife and collected on 200 mesh grids, and double counterstained with uranyl acetate and subsequently lead citrate. The ultrathin sections were examined in a JEOL JEM 1400 transmission electron microscope operated at an acceleration voltage of 80 kV. An Olympus video camera was used to perform images capture.
Results

Preoperatively, blood glucose level was 21.6 mg/dL. Histopathological examination confirmed the diagnosis of functional pancreatic neuroendocrine tumor, classified as ENET–TNM stage: pT1pN0pM0, WHO grade 1. Ki67 was scored as 1%.

Postoperatively, the evolution was uneventful, and blood glucose level was between normal. Surgical resection is curative in almost all cases of benign insulinoma [10, 11]. Preoperative localization of the pancreatic endocrine tumor, including insulinoma is a prerequisite for a successful resection of the tumor.

Electron microscopic investigated control pancreas has a normal architecture as well as normal cell type composition of islets. TEM investigations showed that in normal pancreatic tissue prelevated from the uninvolved, non-tumoral part of the patient pancreas, at least three endocrine cell types (α, β, δ) can be identified (Figures 2 and 3).

Figure 2 – Overview from a normal pancreatic tissue. α, β, δ endocrine pancreatic cells can be identified. N marks a round in shape euchromatic nucleus of a β-cell. Str: Stroma; Exo: A small sector of an exocrine pancreatic cell.

Figure 3 – Three endocrine cell types can be identified: α, β and δ cells inside of a small area of the insulinoma tumor. α-cell exhibit an euchromatic (Nr) nucleolated (nu) nucleus, while β-cells have heterochromatic nuclei (Nc).

The islets were hyperplastic. The neoplastic lesions prevalently represented by β-endocrine cells are extensive, detrimental to pancreatic exocrine tissue areas as well as to other endocrine cell types.

TEM investigated pancreatic tumor showed that ultrastructurally, two main β-cell phenotypes are detected: (1) cells with big and round in shape light euchromatic nuclei and (2) cells with polymorphic in shape and dark heterochromatic nuclei (Figures 4 and 5). The nuclei of both β-cell types have one (seldom two) prominent nucleoli. It seems that in case of phenotype (2) β-cells, the nuclei enter a kind of shrinkage concomitantly with chromatin condensation (Figures 4, 5 and inset in Figure 12) to reach karyorrhexis stage (not showed). Rare endocrine epithelial cells enter and follow apoptotic process (not shown).

Figure 4 – Overview on tumoral endocrine pancreas. Two β-cell types can be identified: cells with euchromatic nuclei (black asterisks), one of them has two nucleoli (black arrows) and cells with heterochromatic nuclei (white asterisks), few of them also nucleolated (white arrows). Heterochromatic nuclei seem to be part of pseudo-syncytia. Almost all β-cells exhibit many polymorphic membraneous emptied vesicles of their specific β-granules. Moreover, extensive edematous areas can be seen inside of β-cells cytoplasm (stars). Bl Vs: Blood vessels.

Figure 5 – Overview shows a pseudo-syncytium realized be two phenotypes of tumor β-cells: cells with heterochromatic (Nc) and nucleolated (nu) nuclei, and cells with euchromatic (Nr) and nucleolated (nu) nuclei. Except for a very short segment of uninterrupted plasma membranes (head arrows), there are no other detectable plasma membranes between β-cells in this field. Heterochromatic nuclei seem to be part of pseudo-syncytia. In the upper part, a blood vessel (Bl Vs) become in close contact with the pseudo-syncytium. WBC: White blood cell; RBC: Red blood cells; EvxRBC: Extravasated red blood cell. Asterisks: Dilated areas inside of tumor β-cells. A lot of polymorphic totally depleted vesicles, except just for few still having specific β-granule content (arrows) can be seen.
The electron microscopic examination of β-tumor cell showed that ultrastructural granules varied considerably. Inside of the β- and α-cells, cytoplasm is depleted or even completely devoid of characteristic β- (insulin-containing granules) and α-cell granules. When compared, cytoplasm of phenotype (1) β-cells with the cytoplasm of phenotype (2) β-cells, results that inside of the cytoplasm of phenotype (2) β-cells there are extensive and more frequently areas devoid of cytomembranary organelles, suggesting to be swollen (maybe edematous), which sometimes fuse with perinuclear cisternal space.

Concerning the small blood vessels, we observed that some of them have a relative small lumen, or are collapsed, mostly because of the large body of endothelial cells projecting inward the lumen. In the uninvolved pancreas of our investigated case, often two basement membranes separate the capillary from the β-endocrine cells (Figure 6). Beta-granules with normal appearance containing insulin near the β-cell plasma membrane, in way to be delivered in close vicinity of normal fenestrated capillary, can be also detected (Figure 6).

The number, size and ultrastructural aspect of the secretory granules vary greatly among the analyzed tissues prelevated from our patient, from cells well granulated containing typical β-granules to cells containing only atypical granules, scarcely or even totally depleted of granular content. Beta-granules composed of one are several angular-shaped cores, suggesting a crystalloid component and a thin membrane surrounding them can be also detected (Figure 7 and inset in Figure 13).

Very seldom ceroid bodies or large lipoid bodies can be observed (not shown).

Concerning the intercellular junctions, mention must be made that, in some areas of tumor, normal desmosomal junctions connect adjacent β-endocrine cells (Figure 7).

![Image 6](image6.png)  
**Figure 6** – A fenestrated (arrows) blood capillary with a continuous basement membrane (white arrows) doubled by another basement membrane (black arrows) interposed between a β-cell and the capillary. At the β cell periphery β-granules can be seen (asterisks), some of them being in way to deliver their content at the extracellular space (black dashed arrows). RBC: Red blood cells.

![Image 7](image7.png)  
**Figure 7** – Three tumor β-cells (β1–β3) with non-altered plasma membranes (black arrows) are junctioned by desmosomes (elliptic areas). Immature as well as mature specific β-granules (white arrows) can be seen.

On the other hand, there are also situations when, for very long distances, no desmosome can be visible between two adjacent β-cells. Moreover, plasma membranes of two adjacent tumor β-cells may be involved in shedding membrane vesicles (Figures 8 and 9). Impaired desmosome figures can be detected (Figure 10). Consequently, severe destruction of plasma membranes takes place (Figure 11).

![Image 8](image8.png)  
**Figure 8** – Shedding vesicles process (squared area) which involve plasma membranes of two adjacent β-cells (β1 and β2), detailed in Figure 9.

![Image 9](image9.png)  
**Figure 9** – Detail of the squared area in Figure 8. Plasma membranes (large arrows) of two adjacent β-cells (β1 and β2) are involved in shedding membrane vesicles (stars).
Relevant infrastructural alterations in a pancreatic neuroendocrine tumor: an insulinoma case

Figure 10 – An illusive desmosome (elliptic area) at the limit between two β-cells (β1 and β2) can be seen. Arrows mark the plasma membranes of the β1 and β2 cells. N: Nucleus.

Figure 11 – Severe destruction (large arrows) of plasma membranes between two adjacent β-cells (β1 and β2). Endoplasmic reticulum appears excessively dilated (stars). Small arrows mark β-granules.

Other abnormalities are recorded: endoplasmic reticulum appears much-dilated (Figure 11). Moreover, quite often, excessively dilatation of the nuclear envelope herniates deeply inside of cytoplasm of β-cells and, few β-granules penetrate inside of cisternal space (Figure 12). Because the limit between tumor endocrine cells is compromised, as is depicted in Figure 13 and inset, β-granules tend to penetrate or even succeeded to penetrate inside of α-cell cytoplasm.

Besides normal microvasculature, there are abnormal microvessels. They are devoid of pericytes and inter-endothelial junctions are precarious. Moreover, endothelial wall appears very thin and fragile, became dehiscent so that, many extravasated white and red blood cells occupy very large areas (Figures 14 and 15). Because of remarkable dissolutions of the tumor β-cells plasma membrane, cytoplasmic content become in direct contact with extravasated red and white blood cells (Figures 14–16). Finally, extensive hemorrhagic area with extravasated blood cells (leakiness) can be seen and the hist架构tecture of the epithelial endocrine tissue appears severely compromised (Figure 17).

Nerve endings were detected in close vicinity of blood capillary inside of the pancreatic stroma between two exocrine areas (Figure 18).
Figure 15 – A fibro-amorphous material (arrows) partially marks the formerly endothelial wall, which was destructured and, consequently, β-cells become in direct contact with the blood cells (RBC). Moreover, a β-cell shows a marked dissolution of the plasma membrane (d β Cell) so that cytoplasmic content become in direct contact with extravasated red blood cells (exv RBC). Bl Vs: Blood vessel; WBC: White blood cell.

Figure 16 – Massive and deeply infiltration of extravasated blood cells (exv RBC) inside of the tumor endocrine β-cells can be seen. Almost all β-cells entered in an advanced state of degradation (d β Cell) becoming in direct contact with extravasated cells.

Figure 17 – A large field of a hemorrhage process showing extravasated blood cells (exv B C) and β-cell remnants (stars). Other few β-cells exhibiting either euchromatic (black asterisk) or heterochromatic shrunk (white asterisk) nuclei with a large number of β-vesicles depleted of their granular content.

Nerve endings are unmyelinated, surrounded by Schwann cell (Figures 18 and 19). Electron microscopic, a nerve ending is represented by few mitochondria and many microvesicles without granular content. Mention must be made that a pericyte is very close to the nerve ending (cca. 200 nm).

Figure 18 – A fenestrated blood vessel (Bl V) and a nerve (Nv) can be seen inside of the stroma between two pancreatic acini of the insulinoma tumor. A pericyte (Pc) is ectopic located because of the nerve location just near the blood vessel.

Figure 19 – Detail for Figure 18. A fenestrated (small white arrows) blood vessel (Bl V) has a basement membrane (white head arrows). Between the blood vessel and the pericyte (Pc) a terminal nerve is enwrapped by Schwann cell (Schw. C). Few black arrows mark the Schwann basement membrane. Few naked axons (white asterisks) with visible neurotubules can be seen. Black asterisk marks a pool of synaptic vesicles. White small arrow marks mitochondria. Black head arrows mark abnormal oriented/distributed subplasmalemmal densities of the dislocated and ectopic located pericyte (Pc) devoid of basement membrane. In inset, small sector of the pericyte (see Figure 19) showing subplasmalemmal densities abnormal oriented/distributed (small black arrows), except just for one (double black arrows) subplasmalemmal density correct located.

Discussion

Five major endocrine cell types are housed in pancreatic islets, each of which produces a different endocrine hormone: alpha cells (α- or A-cells) secrete glucagon, beta cells (β- or B-cells) secrete insulin, delta (δ- or D-cells) secrete somatostatin, PP (or F) cells secrete pancreatic polypeptide, epsilon (ε) cells secrete ghrelin [12, 13].
Pancreatic neuroendocrine tumors are a sub-group of gastroenteropancreatic neuroendocrine tumors [1]. Based on their ability to secrete or not to have the ability to secrete hormones, pancreatic endocrine tumors are classified as functional or non-functional. Non-functional tumors comprise approximately 30% of pancreatic endocrine tumors, and the majority of these will have metastases at presentation. There are very rare reported cases when a non-functional metastatic pancreatic neuroendocrine tumor transformed in an insulin-secreting tumor [14].

Endocrine component of the pancreas represents approximately 1% of the whole pancreatic mass [15], but it plays a crucial role in regulating metabolic function, mainly the glucose metabolism. The concentration of glucose in blood stream is a very important parameter of human body homeostasis. Endocrine tumors account for approximately 1–2% of all pancreatic neoplasms. An insulinoma tumor is made up of β-islet cells that constantly secrete insulin, causing hypoglycemia. An insulinoma is a tumor, usually benign, and is malignant in less than 10% of patients. PNETs grow slower than exocrine pancreatic tumors.

Incidence peak of insulinoma is in adulthood between 40 and 60 years of age, rarely found in children [16]. Our investigated insulinoma case, a 37-year-old male, was hospitalized with a presumptive diagnostic of insulinoma. An overview through endocrine pancreatic insulinoma lesions shows two distinct β-cell types: (1) β-cells with large, round or ovoidal, light euchromatic nuclei and (2) β-cells with polymorphic and dark heterochromatic nuclei. Both euchromatic and heterochromatic nuclei are nucleolated.

On consider that normal process of β-granule maturation start with pale content within a smooth membranous sac, followed by a progressive increase of density and finished with the appearance of crystalline profiles inside of mature granules. The non-crystallized granules are considered an earlier, incompletely mature secretion form. Mention must be made that in spite of the fact that many β cells showed prevalently immature granules, severe hypoglycemia associated to insulinoma, suggests that even immature delivered granules have a hormonal content with a high degree of potency [17, 18].

The number and infrastructure of granules varied greatly among the tumors and even in individual tumor cells. Using electron microscopic examination of a large number of insulinomas, Creutzfeldt et al. [19] described few categories of human insulinomas: (1) tumors with typical β-granules; (2) tumors with typical and atypical β-granules; (3) tumors with atypical granules only; (4) tumors that are virtually agranular [18]. Accordingly with the above classification, our investigated case belongs to the fourth category. Indeed, electron microscopic investigations showed that most of the tumor cells from the insulinoma lesions contain very less β-granules. They are polymorphic, some of them composed of one or several angular-shaped cores, suggesting a crystallloid component, and a thin membrane surrounding them, and their general appearance indicates their β-cell origin. The beta-granules occasionally fused with the plasma membranes of tumor cell or of large and polymorphic intracytoplasmic vacuoles, sometimes entering inside of excessive enlarged endoplasmic reticulum or perinuclear envelope. Our observations suggest the possibility of migration of β-granules into the tissue spaces by holocrine secretion through the breakdown of the tumor cells. Such figures were also described by [18]. This might favorably explain the mechanism for the paroxysmal hypoglycemic crisis [20].

PNETs are either (1) functional tumors (produce hormones that cause symptoms; most are benign) or (2) non-functional tumors (produce hormones that do not cause symptoms; symptoms are caused by the tumor as it grow and spreads). Tumors of the endocrine pancreas are potentially malignant neoplasms Treatment options for pancreatic neuroendocrine cancer are very limited. Surgical removal of the tumor is a common treatment.

Malignant insulinoma is a rare form of cancer. Malignant progression is accompanied by the progressive accumulation of multiple genetic lesions [21]. Malignant metastasizing pancreatic endocrine tumors are slowly growing neoplasms. The prognosis for patients with malignant insulinoma is far better than for patients with more common exocrine pancreatic cancer. Nonetheless, malignant insulinoma remains a cancer with poor prognosis: 5-years survival of 35% is reported [21]. In general, survival from the time of diagnosis of the malignant endocrine pancreas ranges between 2 and 10 years [22]. The overall 5-year survival rate is in the range of 30% in NF-PNETs to 97% in insulinoma, one category of NF-PNET [1].

It is very difficult to discriminate between benign and malignant tumors in case of islet cell pancreatic tumors. So far, there is no specific morphologic, biochemical or genetic trait allowing that. Neuroendocrine tumors of epithelial type are positive cyto keratin [23]. Malignancy is usually associated with the presence of secondary tumors (metastasis). A very important question arises: to which extent, transmission electron microscopic analysis can provide useful information about putative malignant insulinoma development in case of patient with insulinoma diagnosis but still not having metastasis at the time of surgical intervention? Here, we investigate by electron microscopy a case of insulinoma. We underline that, to some extent, some infrastructural abnormalities can be considered to discriminate between benign and malignant insulinoma.

Electron microscopic examination of our insulinoma case revealed few interesting peculiar aspects. First, the neoplastic lesions prevalently represented by β-endocrine cells are extensive, detrimental to pancreatic exocrine tissue areas as well as to other endocrine cell types. In our investigated case, almost all β-endocrine pancreatic tumor cells are devoid of specific β-granules or have an atypically aspect. Most benign insulinomas are rich in insulin-containing cells, whereas in malignant types such cells may contain rare or predominantly atypical β-granules [24].

Plasma membranes and also inner cell cytomembranes (nuclear envelope, endoplasmic reticulum, mitochondria) are very labile and permis sive even for some β-granules, so that large apparently edematous dilatations are visible as can be documented by Figures 11 and 12. There are large fields with tumor cells, which undergo a visible degeneration finishing with partially or totally plasma membrane dissolution. These severe alterations
lead to cell content liberation in the interstitium. Indeed, the most important finding in this report for a case of insulinoma is the high fragility of plasma membrane of tumor cells. As a consequence of that abnormality, plasma membrane of adjacent tumor cells, especially β-cells phenotype (2), tend to perform membrane recombination and shedding membrane vesicles (Figures 8 and 9), plasma membrane dissolutions (Figures 13 and 15) leading to the large pseudo-syncytia formation (Figures 4, 5 and 16). Mention must be made that, interestingly, especially β-cells with heterochromatic nuclei seem to form large pseudo-syncytial figures. Concerning the plasma membrane of some pancreatic tumor β-cells involvement in the process of shedding membrane vesicles now, is well documented that tumor cells are able to shed membrane vesicles [25], considered to modulate the tumor niche through horizontal transfer of bioactive molecules [26]. There is a possible explanation for all above-mentioned infrastructural abnormalities. High plasticity/fragility of cytOMEMBRANES, including plasma membrane is a hallmark for the malignant tumor cells [27–29]. Plasma membrane is a dynamic mosaic of distinct micro-domains often corresponding to specific infrastructures and unique molecular composition, properties and functions. On may speculate that long exposure of endocrine cells to a lot of paracrine factors (cytokines) and enzymes produced by extravasated blood cells, including inflammatory cells, leads to the disorganization of surface domains or even focally plasma membrane destruction, already fragile by itself because of being malignant tumor cells.

Moreover, down regulation of cell–cell and cell– extracellular matrix adhesions might result in aberrant cell behavior, including the promotion of cell migration and consequently invasive growth, a pre-requisite for ectopic places secondary tumor formation (metastasis). Here, we emphasize the power of the microenvironment: as much as the stroma (including blood capillary) vicinity is altered, the adjacent epithelial tissue is affected in their organization and function [28, 30–33]. All above-mentioned ultrastructural abnormalities in our investigated case suggest a possible evolution to a malignant insulinoma.

Besides the fact that tumor cells could have re- differentiate, developing the ability to secrete a hormone (e.g., insulin), not present at diagnosis or at time of tumor resection [14], there is the possibility that an endocrine pancreatic cell (which normally express a specific hormone), exhibiting also another hormone type, detected by immunochemistry/immune electron microscopy. This may happen because the limit between tumor endocrine cells is compromised, as is depicted in Figure 13 and inset, β-granules penetrate inside of α-cell cytoplasm. In a case of somatostatinoma, we observed that, also because of high-fragility of δ-endocrine tumor cell plasma membrane, specific somatostatin granules penetrates inside of adjacent α- or β-cells (unpublished data). Ohneda et al. [34] reported there is also the possibility that a malignant insulinoma transformed into a glucagonoma syndrome.

In order to allow easy hormone transfer across the endothelium and then ready access to the circulation, like other endocrine glands, pancreatic islet cells are richly vascularized by fenestrated microvesicles. Although islets comprise approximately 2% of the pancreatic mass, they receive about 10–15% of the pancreatic blood flow [6]. Many, β-cells face one fenestrated capillary, so that insulin directly enters bloodstream. Not all β-cells are in intimately rapport with blood capillaries so that, there is also possible, that insulin is first secreted into the interstitial space between the β-cells [35].

Normal function of β-cells, including insulin transcription, insulin secretion, as well as β-cell proliferation depends on the basement membrane, which accompany endothelial wall of pancreatic islet microvasculature [36]. In agreement with Lacy and Greider [12] observations, in the uninvolved pancreas of our investigated case, often two basement membranes separate the capillary from the β-endocrine cells (Figure 6). Beta-granules with normal appearance containing insulin near the β-cell plasma membrane, in way to be delivered in close vicinity of normal fenestrated capillary, can be also detected (Figure 6).

Secrected insulin follows a packaging process in small secretory granules, then a trafficking process towards the plasma membrane where they fuse to perform exocytosis in the extracellular space. As we observed, the vast majority of β-cells from extensive insulinoma tumor areas are almost completely degranulated, meaning that the exocytosis is a very active and rapid process. The cellular events preceding exocytosis as well as granule trafficking and, extracellular deliverance of their content remains not yet well understand. Related to the molecular machinery of exocytosis in β-cells, now is postulated that a group of protein referred to as SNARE proteins facilitate exocytosis by bringing the vesicle membrane in close contact with the plasma membrane, but Ca²⁺- dependent exocytosis of secretory granules should be also considered [37, 38].

Usually, vascular endothelium is quiescent in the adult. There are some exceptions, namely some specific situations (e.g., during post-lesional tissue regeneration or during tumor growth) when new blood vessels are formed from pre-existing vasculature, a process known under the name of angiogenesis.

It is well known that to grow beyond few cubic millimeters, a solid primary tumor or new secondary tumors to be formed, angiogenesis is a crucial requirement [28, 39]. Tumor angiogenesis depends on the balance of proangiogenic factors such as vascular growth factor (VEGF) and their inhibitors counterpart as angiotatin, endostatin, etc. [40, 41]. VEGF seems to act in a paracrine manner during both normal pancreas function and neoplastic pancreatic insulinoma [42].

Yamashita et al. [43] showed that in a malignant insulinoma, tumor stroma is very rich in blood vessels. Our electron microscopic investigations showed that, very often, inside of the endocrine pancreatic tumor, many microvessels are detected. Some of them look normal, having a fenestrated wall with a continuous basement membrane around. Greider et al. [44] also reported that in a case of pancreatic β cell tumor, endothelial cells of the capillaries in the non-neoplastic islets were normal. Mention must be made that many other microvessels are abnormal. They are devoid of pericytes, inter-endothelial junctions are precarious. Associated pericytes to the
capillary endothelia is a hallmark of stable microvasculature, limiting endothelial cell proliferation. Loss of pericytes can lead to (a) endothelial cell hyperplasia [15] and (b) new but weakness microvasculature formation as it happens in tumor angiogenesis [28, 32]. Impairment of endothelial wall-endocrine cell interactions contributes to tumor invasion and metastasis [45]. Absence of tumor pericytes is associated with increased tumor metastasis [15]. Indeed, Xian et al. [46] showed that β-cell tumors that are deficient for neural cell adhesion molecules (NCAM) increased vessel leakiness and re-expression of NCAM prevented metastasis by pericytes reintegration into periendothelial space. Moreover, in the present study, endothelial wall appears very thin and fragile, so that many extravasated white and red blood cells can be seen. Sometimes, the extravasated cells, occupy very large areas, so that the histotarchitecture of the epithelial endocrine tissue appears severely compromised (Figures 14–17).

Like in human population, spontaneous tumors of pancreatic islet cell origin are rare in animals, including rats [47]. There are some chemicals capable to induce islet cell adenomas in rat (mostly of β-origin). Repeated intravenous administration of 4-hydroxyaminoquinoline-1-oxide (4HAQO) induce 4HAQO-DNA adduct formation, and consequently islet cell tumors of β-cell origin in rats at high incidence [48]. Electron microscopic examination of such experimentally pancreatic tumors induced with 4HAQO revealed that to some extent, mimic the ultrastructural aspects encountered in human insulinoma. Like in human insulinoma, in rat endocrine pancreatic lesions, in spite of the fact that endoplasmic reticulum and Golgi apparatus are present, β-cells exhibit scanty granules or are almost agranular (they appear devoid of granular content) suggesting an active secretion but a rapidly depletion of the insulin content [48].

Frequently observed tumor β-cells with euchromatic nuclei suggest active cells, may be the reservoir of proliferative tumor cells.

Mention must be made that rare tumor cells are involved in atypical mitosis (not shown).

The pancreas is innervated by sympathetic and parasympathetic neurons; signals transmitted by adrenergic and cholinergic nervous fibers clearly modulate islet cells and their hormone secretions [6, 12, 13]. Nerve ending were detected inside of the pancreatic stroma between two exocrine areas. Nerve endings are unmyelinated surrounded by Schwann cell. Electron microscopic a nerve ending is represented by few mitochondria and many microvesicles without granules.

In Figures 18 and 19, a slender cell extension can be seen. Specific infrastructure represented by subplasmalemmal densities [49] for a pericyte are visible but, mention must be made that, except just for one subplasmalemmal density correct located, at the abluminal face of pericyte (double black arrows in inset Figure 19), the other two subplasmalemmal densities are abnormal distributed meaning at the luminal face of pericytes (small black arrows, in Figure 19).

A tumor is a complex multi-cellular ecosystem represented by (1) neoplastic genetic altered cells and (2) tumor stroma represented by (a) different cell types (fibroblasts, fibrocytes, mast cells, inflammatory cells, endothelial cells, pericytes, naked or myelinated nerves, etc.) as well as (b) extracellular matrix (basal lamina, collagen and elastic fibers and soluble molecules). Autocrine and paracrine factors offer the necessary support for tumor growth, a permanent cross talk between malignant cells and peritumoral stroma being necessary [32].

**Conclusions**

Electron microscopic investigations of a neoplastic insulinoma revealed severe alterations of cell–cell and cell–extracellular matrix (ECM) junctions and, a high fragility of plasma membranes of β-tumor cells, leading to pseudo-synctia formation. Moreover, also inner cell cytomembranes (nuclear envelope, endoplasmic reticulum, mitochondria) exhibit excessive dilatations. Many small blood vessels are very fragile, become dehiscent, delivering inflammatory cells so that, large areas of epithelial tumor cells are degraded. Altogether above-mentioned alterations leading to the abolishment of cell–cell and cell–ECM control might result in aberrant β-tumor cell behavior, as high degree of cell movement, including intravasation and, eventually, second tumor formation.

**Acknowledgments**

The study was funded by Project No. RO1567-IBB07/2013 from the Institute of Biology Bucharest, Romanian Academy.

**References**


Corresponding author
Nicolae Mirancea, Professor, PhD, Senior Scientist Grade I, Institute of Biology Bucharest of Romanian Academy, 296 Independenței Avenue, 060031 Bucharest, P.O. Box 56–53, Romania; Phone +4021–223 90 72, e-mail: nick_mirancea@yahoo.com

Received: October 21, 2013
Accepted: July 3, 2014