Cytological and immunocytochemical characterization of the insulin secreting insulinoma cell line RINm5F

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Summary. The rat insulinoma cell line RINm5F, an insulin secreting pancreatic beta cell line, has been used as an attractive model for basic studies of the mechanisms of insulin secretion and, more recently, as a model for the development of alternative methods for the treatment of diabetes. To elucidate the cytological properties and expression patterns of hormones of the gastro-entero-pancreatic system, suspensions of RINm5F cells were investigated by various methods including immunocytochemistry on serial semithin sections, quantitative immunocytochemistry, routine electron microscopy, immuno-electron microscopy, in situ hybridization, and TUNEL technique. At the ultrastructural level, several phenotypes of RINm5F cells were characterized by differences in the number, shape, size, and density of their secretory granules. The most common type contained a mixture of round granules varying in size and electron density. A second type predominantly contained relatively large, moderately dense granules. Moreover, a minority of cells was characterized by the occurrence of polymorphous electron dense granules or the complete absence of any secretory granules. The immunohistochemical data showed that, among the established islet hormones, insulin was present in more than 50% of cells, whereas glucagon and somatostatin occurred only sporadically. Though cells positive for pancreatic polypeptide (PP) were not found, PP-related peptides (NPY and PYY) however could be detected in a minority of cells. The great majority of RINm5F cells were immunoreactive for chromogranin B (CgB), followed by insulin, chromogranin A (CgA), and serotonin (5-HT). In addition to intercellular differences in the density of immunostaining, numerous colocalizations of immunoreactivities were found, suggesting that RINm5F cells represent a mixture of subtypes concerning the individual pattern of hormone expression. The present results reveal a wide range of heterogeneity with respect to the morphology and especially the hormone content between individual RINm5F cells.

Introduction

Beta (B-) cells are found exclusively in the pancreas, where they secrete insulin in response to glucose stimulation. Destruction of these cells or failure of the body to utilize insulin properly results in diabetes, characterized by high blood sugar levels. The initiation and progression of this auto-immune cell destruction, however, remain unclear. One approach to treating diabetes is transplantation of either the pancreas or isolated islets of Langerhans (for review see Titus et al., 2000; Fontaine and Fan, 2003; Oberholzer et al., 2003). Major obstacles to this approach are immunologic rejection, the poor availability of donor material, and the poor yield of islets during the isolation procedure. To generate an unlimited supply of insulin producing cells for transplantation or for biochemical and molecular biological research, numerous investigators have attempted to establish insulin producing cell lines termed RIN, HIT, MIN6, INS-1 and BRIN cells (for review see Poitout et al., 1996; McClenaghan and Flatt, 1999). More recently, research has focused on bio-engineering insulin-secreting cells from stem cells (for review see Roche et al., 2003). The rodent insulinoma cell lines have proved useful in studies of B-cell biology because they share characteristics with normal B-cells. However, compared to normal B-cells of the pancreas, these cell lines are largely devoid of an insulin-secretory response to physiological glucose stimulation.
One of the most widely used insulin secreting cell lines for studying the mechanisms of insulin secretion and molecular events underlying B-cell function and dysfunction is the RIN-cell clone RINm5F (Gazda et al., 1980; Bhathena et al., 1984). This immortalized cell line was originally derived from a radiation-induced transplantable rat islet cell tumor (Chick et al., 1977). In the past 20 years, no comprehensive morphological characterization of this widely used cell line has been published apart from only a few histological data concerning the shape, secretory granules, and ultrastructure of corresponding cells (Gazda et al., 1980; Hofliezer et al., 1987). RINm5F cells exhibit an intact insulin gene but have lost the ability for glucose stimulated insulin secretion after in vitro culture (Bhathena et al., 1984; Poitout et al., 1996). They are characterized by a defective glucose phosphorylating enzyme glucokinase due to a relative lack of the glucose phosphorylating enzyme glucokinase (Lenzen and Tiedge, 1994).

Recently, in order to generate surrogate B-cells exhibiting glucose-sensitive insulin secretion, attempts have been directed toward methods for genetically engineering B-cell lines (for review see Docherty, 1997; Ferber et al., 1997; Soria et al., 2000). Using the clonal RINm5F cell line as a model, approaches have been made to bioengineer surrogate B-cells for insulin replacement therapy in insulin-dependent diabetes mellitus, and a RINm5F cell line exhibiting the overexpression of glucokinase and the glucose transporter GLUT2 was generated (Tiedge et al., 2000). The physiological glucose responsiveness of this glucokinase transfected RINm5F cell line could also be demonstrated in vivo after implantation into diabetic rats (Tiedge et al., 2000). In the RINm5F clones used for these investigations, we have studied the cytology and cytochemistry of normal non-transfected RINm5F control cells and present a detailed description of light- and electron microscopical findings concerning the cell structure and particularly the pattern of hormones expressed in the RINm5F cell line. The aim of this investigation was to demonstrate by morphological analysis possible alterations or differences in this insulinoma cell line compared with normal pancreatic B-cells and to survey by immunohistochemistry the hormones and markers known to occur in endocrine cells of the gastro-entero-pancreatic system on the basis of serial semithin sections (Grube and Kusumoto, 1986).

Materials and Methods

Cell preparation and embedding

Cells of the insulinoma cell line RINm5F were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Growing RINm5F cells from early passages were trypsinized and harvested in PBS. Pellets were prepared from the cell suspensions (cell count: 10^6 cells/tube) by spinning down at 1000 rpm for 10 min.

Light microscopy

Pellets were snap frozen in isopentane precooled with liquid nitrogen, freeze-dried at -35°C for 72 h, and fixed by vapor phase p-formaldehyde or di-ethylpyrocarbonate (for details, see Grube and Kusumoto, 1986). Following embedding of the specimens in epoxy resin (Araldite), serial semithin sections were cut at 0.5 µm and mounted individually on microscopic slides. Routine staining was performed by Azur II and methylene blue (Richardson et al., 1960).

For application of the in situ hybridization technique and analyses of apoptosis, sedimented RINm5F cells were resuspended in a fixative containing 4% p-formaldehyde and 0.1 M phosphate buffer (pH 7.4) for 2 h. After washing in 0.1 M phosphate buffer and dehydration by ethanol with respective 5 min centrifugation at 1000 rpm, the cells were embedded in paraffin.

Routine electron microscopy

RINm5F cells were fixed in a solution of 2% glutaraldehyde and 2% p-formaldehyde in 0.1 M cacodylate-HCl buffer (pH 7.3) for 2 h. After postfixation in 2% osmium tetroxide for 1 h at 4°C, the cells were embedded in Epon 812. Ultrathin sections were cut at 50 nm on an ultramicrotome Ultracut (Leica, Nussloch, Germany), mounted on copper grids, and contrast-stained with uranyl acetate and lead citrate. The sections were examined with a Zeiss EM 10 electron microscope.

Immunohistochemistry

Antisera

The polyclonal antisera used in the present study are listed in Table 1. Most of the antisera have been characterized and employed in our own previous investigations. For the immunoelectron microscopy of insulin, the antisera LAA was determined to be more suitable than antisera Y370.

Immunohistochemical protocol (light microscopy)

Semithin serial sections of Araldite embedded RINm5F cells were sequentially immunostained for the established islet hormones, other peptides, and amines known to occur
in the endocrine pancreas (see Table 1). Since about 10–20 semithin sections pass through the same cells, colocalization of immunoreactivities could be unequivocally attributed to cells identified in adjacent sections.

Immunostaining of semithin plastic sections was performed as described by Grube and Kusumoto (1986). Briefly, after removal of the resin by sodium methoxide, the sections were immunostained by the avidin–biotin–peroxidase complex (ABC) technique (Hsu et al., 1981). Following incubation with 4% non-immune goat serum (30 min), the sections were incubated in appropriately diluted first antisera (24 h at 4°C). Subsequently, biotinylated goat anti-rabbit IgG (1:100; 30 min) and a preformed streptavidin/biotin-peroxidase complex (30 min; both obtained from Jackson Immuno Research, West Grove, USA) served as the second and third layer of the protocol (final concentration of 5 μg/ml of streptavidine and 0.7 μg/ml of biotin-peroxidase). Peroxidase activity was demonstrated by a solution containing 0.7 mM diaminobenzidine (DAB) and 0.002% H2O2 in 0.01M PBS buffer, pH 7.3. The sections were mounted without counterstaining and were examined in an Orthoplan microscope (Leitz, Wetzlar, Germany) by brightfield illumination or phase-contrast optics. Specificity controls to exclude methodological or antibody nonspecificities were performed as recommended in the literature (see Table 1; Grube and Kusumoto, 1986; Sternberger, 1986).

**Quantification**

The relative frequency of RINm5F cells exhibiting immunoreactivities for the antigens proved in this study was determined in serial semithin sections at a microscopical magnification of 1:250 (Table 2). Immunoreactivities for insulin, serotonin, and chromogranins A and B were more closely determined by computer-assisted analysis (for methodical details see: Redecker and Bargsten, 1993). Briefly, quantitative measurements were performed with the interactive image analyzing system IBAS (Zeiss-Kontor; Munich, Germany) equipped with a Photomicroscope II (Zeiss) and a video camera CCD/89D (Sony). To standardize the measurements, the system was calibrated by adjusting the amount of light transmitted through the section to a constant gray value of 170 (gray value range was 0 = black to 255 = white). The images of cells to be quantified were entered into the

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Source</th>
<th>Working dilution</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin Y370 (INS)</td>
<td>Yaishihara Institute Shizuoka, Japan</td>
<td>1:20,000</td>
<td>-</td>
</tr>
<tr>
<td>Insulin LAA</td>
<td>Novo, Bagsvaerd, Denmark</td>
<td>1:1,000</td>
<td>1</td>
</tr>
<tr>
<td>Glucagon (GLU)</td>
<td>Dakopatts, Hamburg, FRG</td>
<td>1:5,000</td>
<td>1</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>H. Eitzrodt, Ulm, FRG</td>
<td>1:4,000</td>
<td>1</td>
</tr>
<tr>
<td>Bov. PP</td>
<td>Dr. R.E. Chance, Indianapolis, USA</td>
<td>1:10,000</td>
<td>1</td>
</tr>
<tr>
<td>NPY</td>
<td>Peninsula, Belmon, USA</td>
<td>1:8,000</td>
<td>-</td>
</tr>
<tr>
<td>PYY</td>
<td>Peninsula, Belmon, USA</td>
<td>1:4,000</td>
<td>-</td>
</tr>
<tr>
<td>Serotonin (5-HT)</td>
<td>Immuno Nuclear, USA</td>
<td>1:20,000</td>
<td>-</td>
</tr>
<tr>
<td>VMAT1</td>
<td>Chemicon, Temecula, USA</td>
<td>1:500</td>
<td>-</td>
</tr>
<tr>
<td>CGA</td>
<td>H. Winkler, Innsbruck, Austria</td>
<td>1:2,000</td>
<td>2</td>
</tr>
<tr>
<td>CGB</td>
<td>H. Winkler, Innsbruck, Austria</td>
<td>1:2,000</td>
<td>2</td>
</tr>
<tr>
<td>SGII</td>
<td>H. Winkler, Innsbruck, Austria</td>
<td>1:500</td>
<td>2</td>
</tr>
<tr>
<td>Pancreastatin (PST)</td>
<td>Peninsula, Belmon, USA</td>
<td>1:2,000</td>
<td>3</td>
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<tr>
<td>Glucokinase</td>
<td>M. Tiedge, Hannover, FRG</td>
<td>1:200</td>
<td>4</td>
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<tr>
<td>GLUT2</td>
<td>WAK-Chemie, Bad Homburg, FRG</td>
<td>1:4,000</td>
<td>5</td>
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</tbody>
</table>

Abbreviations: Bov. PP = bovine pancreatic polypeptide; CGA = rat chromogranin A; CGB = rat chromogranin B; GLUT 2 = glucose transporter; NPY = neuropeptide Y; PYY = peptide YY; SGII = secretogranin II; VMAT1 = vesicular monoamine transporter.

Table 2. Synopsis of the relative frequencies of immunoreactivities in RINm5F cells as observed in immunostained semithin sections

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Relative frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>+++ / +++</td>
</tr>
<tr>
<td>Glucagon</td>
<td>- / +</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>- / +</td>
</tr>
<tr>
<td>Bov. PP</td>
<td>-</td>
</tr>
<tr>
<td>NPY</td>
<td>+ / ++</td>
</tr>
<tr>
<td>PYY</td>
<td>+</td>
</tr>
<tr>
<td>Serotonin</td>
<td>+</td>
</tr>
<tr>
<td>VMAT1</td>
<td>++</td>
</tr>
<tr>
<td>CgA</td>
<td>+ / ++</td>
</tr>
<tr>
<td>CgB</td>
<td>+++</td>
</tr>
<tr>
<td>SgII</td>
<td>- / +</td>
</tr>
<tr>
<td>Pancreastatin</td>
<td>+ / ++</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>-</td>
</tr>
<tr>
<td>GLUT2</td>
<td>+ / ++</td>
</tr>
</tbody>
</table>

Symbols: +++ = majority of cells; ++ = about 50% of cells; + = few cells; - = none
Abbreviations: see Table 1

system and displayed at a final magnification of 1:1,200. Using a measuring program (macro “areabar”) which we had developed in cooperation with the supplier, the total area occupied by immunoreactive cells (object area) was quantified automatically. Quantitative determination was done in 20 immunostained semithin sections. In each of the sections, 10 different fields were selected randomly for measuring. The field area (18,950 μm²) was coincident with the area displayed on the monitor. To discriminate between the object and background, the thresholds for the background were separately determined in each section before measuring by a densitometric procedure. Only gray values below the thresholds of 145–155 were detected by the system. The measuring data, corresponding to the total area of immunoreactive RINm5F cells in the measuring field, were expressed as the percentage of the total field area. Significant differences were determined by the Kruskal-Wallis test.

Immunohistochemical protocol (electron microscopy)

For immuno-electron microscopy, RINm5F cells were fixed in a solution containing 4% p-formaldehyde and 0.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.3; 2 h). Following dehydration in graded alcohols, the cells were embedded in Epon 812. Ultrathin sections were cut at 70 nm and mounted on nickel grids. Before immunolabeling, the sections were etched (30 sec) with sodium methoxide diluted 1:30 in a mixture of equal parts of methanol and benzene. After incubation in 5% non-immune goat serum (20 min), the sections were incubated at 4°C overnight with the following antibodies: anti-insulin (1:500), anti-serotonin (1:1,000–1:2,000), anti-chromogranin A (1:250), and anti-chromogranin B (1:250). They were then treated with gold-labeled goat anti-rabbit IgG (size of gold particles: 10 nm) diluted 1:20–1:40 for 1 h at room temperature. Between each step of the protocol and for dilution of the antisera, 0.02 M Tris-HCl buffer, pH 7.6, containing 0.1% BSA was used. Following the immunoreactions, the sections were contrast-stained with uranyl acetate and examined in a Zeiss EM 10 electron microscope.

TUNEL assay

To detect cells undergoing apoptosis, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nickend labeling (TUNEL) technique (Gavrieli et al., 1992) was employed in a modified manner (Bode et al., 1997). In brief, after the removal of paraffin and washing (0.05% Tween in Tris-buffered saline), the sections were incubated with FITC-labeled dUTP (1.7 nmol/l) and a TdT buffer solution containing TdT (12.5 U), potassium cacodylate (200 mmol/l), Tris-HCl (25 mmol/l), BSA (1.25 mg/ml), and cobalt chloride (5 mmol/l) for 70 min at 37°C. After wash-

Fig. 1. Light (a) and electron micrographs (b–d) of RINm5F cells. a: The cells are generally round in shape but differ evidentively in size. Mitotic stages (arrows) and binucleated cells (asterisk) are visible. ×1,100. b–d: Ultrastructure of RINm5F cells and their secretory granules. b: The cells show intercellular differences regarding number, size, and shape and electron density of their secretory granules. Individual cells characterized by relatively large granules of varying density (arrows) or the complete absence of granules (asterisk) are shown. ×4,400. c: The most frequent cell type is seen here to contain a mixture of round granules, which differ in size and density. ×9,500. d: Sporadic cells are characterized by numerous polymorphous small-sized secretory granules (asterisk). ×8,800
Fig. 1. Legend on the opposite page.
ing, the incorporated dUTP was revealed immunocytochemically by an anti-FITC primary antibody, with a bridging
anti-mouse IgG (1:50) as secondary antibody and the subsequent application of an alkaline phosphatase-anti-alkaline
phosphatase (APAAP) complex (1:50) and a fast blue containing substrate solution as a chromogen for visualization.
P-formaldehyde-fixed cryostat sections of rat thymus (containing a substantial percentage of apoptotic T cells) served
as positive controls.

In situ hybridization

A specific oligonucleotide probe reverse complement of bases 4260-4288 of the rat insulin I gene (Soares et al., 1985) was synthesized and labeled with FITC (Biognostik; Göttingen, Germany). A randomer with matched length and CG-content (Biognostik) served as negative control. Deparaffinized sections of paraffin-embedded RINm5F cells were pretreated with a solution of 10 μg/ml proteinase K in 0.1 M Tris-HCL buffer (pH 8) for 20 min at 37°C. After post-fixation with 0.4% paraformaldehyde in 0.1 M PBS (pH 7.2) for 20 min at 4°C and short washes with 2× standard saline citrate (SSC), the hybridization solution was applied to the sections which contained 60 pmol/ml of the oligonucleotide probe diluted in HybriBuffer (Biognostik). Hybridization was performed by overnight incubation at 37°C, followed by posthybridization washes with 1× SSC at room temperature for 2×5 min and 0.1× SSC at 40°C for 2×15 min. Hybridized oligonucleotide probes were detected with anti-FITC Fab-fragments conjugated with alkaline phosphatase (DAKO, Hamburg) using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrates.

Results

Cytology

RINm5F cells, immediately fixed after removal from the culture dishes, appeared round and varied considerably
in size (mean diameter about 10 μm; Fig. 1a). Occasionally, cells with short cytoplasmic processes as well as large-
sized cells with a diameter of up to 25 μm were present. The nucleus was usually oval or roundish and contained
1–2 nucleoli within an euchromatin-rich nucleoplasm (Fig. 1b, c). In some cases, binucleated cells and cells exhibiting
mitotic stages occurred (Fig. 1a). The cytoplasm was relatively pale without remarkable intercellular differences.
Even at the light microscopical level, vesicular and granular structures could be seen in some cells. Ultrastructurally,
the cytoplasm of RINm5F cells contained specific secretory granules (Fig. 1b). They were mainly localized in the cell
periphery. With respect to the size, shape, and electron density of these secretory granules, RINm5F cells showed
intercellular differences. Most cells contained a mixture of round granules differing in size and electron density (Fig.
1c). Other cells predominantly exhibited relatively large and roundish granules with a moderately dense content
(Fig. 1b). A minority of cells was characterized by the occurrence of polymorphous electron dense granules (Fig.
1d). Some cells completely lacked secretory granules (Fig. 1b).

Other cell organelles such as the Golgi apparatus and rough endoplasmatic reticulum displayed no peculiarities apart
from minor intercellular differences in their amount and arrangement. The mitochondria, however, showed intercell-
ular differences in their number, shape and size. In numerous cells, large-sized mitochondria occurred in addition
to relatively small mitochondria. Furthermore, bundles of filaments were visible in many cells.

Occasionally cells were found obviously undergoing cell death. They were characterized by pale nuclei, ruptured
membranes and a prominent cytoplasmic vacuolization. However, typical ultrastructural features of apoptosis—
condensation, fragmentation and margination of chromatin towards the nuclear membrane —were not observed.

Application of the TUNEL assay revealed that apoptotic cells occurred very rarely in the culture passages used in
this study. The number of TUNEL-labeled RINm5F cells was estimated at less than 1 percent.

**Fig. 2.** Proof of pancreatic hormones and GLUT2 by immunohistochemistry and in situ hybridization for insulin. a–c: Three semithin sections are immunostained for insulin (a), glucagon (b), and somatostatin (c). Numerous cells show immunoreactivities for insulin, while cells immunoreactive for glucagon or somatostatin occur very rarely. Phase contrast. a: ×530, b and c: ×1,000. d: Immuno-electron microscopy for insulin. Immunostaining of secretory granules shows differences in the density of labeling. ×40,000. e: Semithin section immunostained for GLUT2. Immunoreactivities occur along the plasma membrane and in single spots within the cytoplasm (arrows). ×530. f and g: Insulin immunoreactivities (f) and in situ hybridization (g) for insulin carried out on paraffin embedded RINm5F cells. Compared with insulin containing cells (f; arrows), more cells display hybridization signals for insulin mRNA (g). ×420
Fig. 2. Legend on the opposite page.
Immunohistochemistry

Islet hormones

Of the established islet hormones, insulin was found most frequently in RINm5F cells (Fig. 2a, f). The staining densities for insulin, however, varied considerably. Numerous cells lacked any immunoreaction for insulin. However, in situ hybridization for insulin revealed that the majority of cells displayed hybridization signals (Fig. 2g). At the ultrastructural level, insulin immunoreactivity was localized exclusively in the secretory granules. Of the various granule types, the roundish secretory granules were predominantly immunolabeled (Fig. 2d). In cells containing polvomorphous granules, no insulin immunoreactivity was seen. Concerning other islet hormones, immunoreactivities for glucagon (Fig. 2b) and somatostatin (Fig. 2c) were found, but only sporadically.

GLUT2 glucose transporter and the glucose-phosphorylating enzyme glucokinase

Numerous RINm5F cells displayed immunoreactivities for the GLUT2 glucose transporter—though, with obvious intercellular differences in the pattern and density of immunostaining (Fig. 2c). In faintly stained cells, GLUT2 immunoreactivity was restricted to the plasma membrane, whereas in cells with dense immunostaining additionally stippled immunoreactivities within the cytoplasm were visible. The glucose-phosphorylating enzyme glucokinase, however, was undetectable in any of the RINm5F cells (not shown).

Hormones of the pancreatic polypeptide (PP) family

Of the peptide hormones of the PP family, immunoreactivities for PP were found in none of the RINm5F cells, and only a small number of RINm5F cells displayed NPY immunoreactivities of varying densities. Likewise, PYY immunostaining was observed very rarely and was restricted to cells which concomitantly exhibited immunoreactivities for NPY. The colocalization of NPY and insulin, however, was never found.

Serotonin and amine transporter

RINm5F cells contained only sporadic immunoreactivities for serotonin with intercellular differences in the density of staining (Fig. 3b). The quantitative determination of immunoreactivities revealed that serotonin immunoreactive cells occurred at the rate of 1:10 compared with the frequency of insulin positive cells (Fig. 3a). Serial sections alternatingly stained for serotonin and insulin showed both colocalized in individual cells. However, RINm5F cells strongly stained for serotonin were usually unreactive for insulin (Fig. 3a, b). At the ultrastructural level, labeling for serotonin was predominantly found in cells containing a mixture of small roundish and oval granules. The individual secretory granules, however, exhibited differences in the density of labeling (Fig. 3c).

After immunostaining for the vesicular monoamine transporter VMAT1 (Fig. 3d), an obviously greater number of immunoreactive cells was found than after staining against serotonin. The VMAT1 immunoreactivities differed in density from strong to very faint. All serotonin positive cells concomitantly exhibited VMAT1 immunoreactivities, whereas most of the VMAT1 containing cells lacked any immunoreactivity for serotonin.

Chromogranins and pancreastatin

Concerning immunoreactivities for chromogranins, the frequency of immunoreactive RINm5F cells as well as the intensity of staining depended on the chromogranin antisera applied. According to our quantitative determinations, CgB immunostained cells occurred more frequently than cells immunoreactive for CgA (Fig. 5). CgA immunoreactivities were mostly of low intensity and were located only in a minority of cells (Fig. 4b), whereas the antisera against CgB revealed an intense immunostaining in numerous cells (Fig. 4c). Examination of sequentially immunostained sections showed that CgA and CgB were generally colocalized with insulin (Fig. 4a-c). Immunoreactivities for SgII, however, could not be detected apart from a very few faintly stained cells. Furthermore, we found immunoreactivities for pancreastatin with intercellular differences in the density of staining in a minority of RINm5F cells (not shown).

Fig. 3. Immunoreactivities for serotonin and VMAT1 in RINm5F cells. a and b: Two adjacent semithin sections were immunostained for insulin (a) and serotonin (b). Only some cells show immunoreactivities for serotonin in colocalization with insulin (arrowheads), or in cells devoid of insulin (arrows). ×660. c: Immuno-electron microscopy for serotonin. Individual secretory granules differ in their density of labeling. ×25,000. d: Semithin section immunostained for VMAT1. Compared with the number of serotonin containing cells in b, more cells obviously display VMAT1 immunoreactivities with intercellular differences in the staining. ×530
Fig. 3. Legend on the opposite page.
The antiserum against CgB was used for immunoelectron microscopical proof of chromogranin immunoreactivities. The labeling by immunogold particles for CgB was heterogeneously distributed in the secretory granules but was not confined to a special type of granule (Fig. 4d). Cells containing polymorphous granules, however, predominately exhibited the most densely labeled granules (Fig. 4e).

**Colocalization of immunoreactivities in RINm5F cells**

Of the 13 immunoreactivities found in RINm5F cells previously described (see also Table 2), we examined 11 for their possible concomitant presence in individual cells by appropriate 'sequential' immuno-staining of serial semithin sections. It was thus able to show that all insulin-immunoreactive cells contained 1 or 2 other immunoreactivities, mainly serotonin or chromogranins (Fig. 6). Curiously, a minority of cells showed a concomitant content of insulin and glucagon. Most of the remaining (insulin negative) cells contained serotonin together with 1 or 2 other immunoreactivities.
Discussion

The present study describes the clonal RINm5F tissue culture cell line frequently used as a model for insulin secreting studies in experimental diabetology. Our studies were focused on the morphological and cytochemical characterization of early-passage nontransfected RINm5F cells. This insulinoma cell line, originally derived from a transplantable islet cell tumor induced by high-dose x-irradiation in an inbred NEDH (New England Deaconess Hospital) rat (Chick et al., 1977), exhibits differences in both structure and hormonal characteristics compared with B-cells of the islets of Langerhans.

Morphology

The insulin secreting B-cells are the predominant cell type in the rat endocrine pancreas. They represent polygonal cells and show a typical localization within the islets, where they predominantly form the medullary region of the islets (Ferner, 1952; Grube et al., 1983; Jörns, 1994). The insulin secreting RINm5F cells growing as epithelial cells develop typical cytoplasmic processes during cell culture (Gazdar et al., 1980). Treatment of RINm5F cells with nerve growth factor (NGF) even leads to a neurite-like extension of the cellular processes (Sher et al., 1995). After trypsinization and removal from the culture, the suspended RINm5F cells tend to become roundish with only small microvilli-like cytoplasmic processes visible at the ultrastructural level.

![Fig. 5. Quantitative determinations of immunoreactivities for insulin, serotonin, CgA, and CgB in RINm5F cells in 20 immunostained semithin sections. In each section the immunostained area of 10 randomly selected measuring fields was calculated. The measuring data, expressed as percentages of the field area (mean ± SD), correspond to the relative frequency of RINm5F cells immunoreactive for these antigens. All immunoreactivities were significantly different from each other, with the highest value for CgB and the lowest for serotonin (p = < 0.001).](image)

![Fig. 6. Schematic presentation of the concomitant content of immuno-reactivities in individual RINm5F cells as found in immunostained serial semithin sections. Boxed areas refer to the corresponding immunoreactivities, and horizontal lines represent concomitant localization of immunoreactivities in individual cells. According to their immunocytochemical staining pattern, 14 different "phenotypes" of RINm5 cells could be distinguished.](image)
The observed variability in size of the cell nuclei may depend on differences in the number of chromosomes. In this respect, the formation of single RINm5F giant cells is probably caused by polyploid nuclei. As reported by Gazdar et al. (1980), the RIN-m cell line had a hypodiploid chromosome number (37–40) at both early and late culture passages. The most conspicuous differences with normal B-cells, however, are related to the ultrastructure of the specific secretory granules. Mature B-cell granules of islets are characterized by a round dense core which is separated from the granule membrane by a wide, clear halo. Immunelectron microscopically, insulin is confined to the core region after processing by proteolytic cleavage from proinsulin along the secretory pathway (Bendayan, 1989; Malide et al., 1995; Molinete et al., 2000). The secretory granules of RINm5F cells, however, have lost the typical appearance of insulin containing B-cell granules and represent a mixture of morphologically different secretory granules containing various hormones. As shown immuno-electron microscopically, insulin-labeling was restricted to cells containing roundish secretory granules, whereas the only rarely occurring cells with polymorphous granules did not contain insulin. These cells resembled enterochromaffin (EC-) cells of the gastro-intestinal system.

**Islet cell hormones and enzymes of the glucose-sensing mechanism**

According to our immunohistochemical findings, about 50 % of the RINm5F cells exhibited insulin immunoreactivities of varying density. The application of in situ hybridization led to positive hybridization signals for insulin in the great majority of the cells, indicating the ability for insulin synthesis in most RINm5F cells regardless of the individual insulin content. However, compared with native rat B-cells, the insulin content in RINm5F cells was clearly decreased (Praz et al., 1983). Weak immunostaining or the total absence of insulin immunoreactivities might be caused by a low number of insulin containing granules and the low insulin content of the granules. The occurrence of poorly or non-granulated cells was confirmed by our electron microscopical studies. For the other pancreatic hormones, glucagon and somatostatin immunoreactivities were very rarely found. These findings correspond to biochemical data indicating small amounts of somatostatin and glucagon in the supernatant fluids of cultured RIN-cell clones (Gazdar et al., 1980; Bhatthena et al., 1982, 1984). However, whether these islet hormones were stored in granules different from those containing insulin or were colocalized with insulin in identical granules remains unclear.

In regard to an essential role of the enzymes in glucose-induced insulin secretion (Lenzen and Tiedge, 1994; Schuit, 1997; Thorens et al., 2000), RINmF cells showed evident differences from normal islet B-cells. A defective glucose-recognition apparatus detectable by an underexpression of the specific glucose transporter GLUT2 and the glucose-phosphorylating enzyme glucokinase (GK) made this insulinoma cell line attractive as an experimental model for the modulation of glucose-stimulated insulin release. Treatment of the RINm5F cell line with sodium butyrate (Tiedge and Lenzen, 1996) and engineering with GLUT2 or GK genes (Tiedge et al., 1993, 1999; Thorens et al., 2000) restored the glucose responsiveness of insulin secretion even at physiological glucose concentrations (Tiedge et al., 2000). The biochemical data revealing the underexpression of GLUT2 and GK in RINm5F cells were largely in agreement with our immunohistochemical findings. GLUT2 immunoreactivities were detectable in numerous cells but showed intercellular differences, whereas glucokinase immunoreactivities were completely absent. GLUT2 immunostaining was visible predominantly on the cell membrane and corresponded to the subcellular localization found in pancreatic B-cells (Sato et al., 1996) as well as in B-cells of islet grafts (Kohmert et al., 1997). Single RINm5F cells additionally exhibited a cytoplasmatic pool of GLUT2, which was also noted for B-cells of whole pancreas grafts (Jörns and Klemmna, 1995).

**Chromogranins and related peptides**

Chromogranins (Blaschko et al., 1967) are large acidic proteins originally detected in secretory granules of the adrenal medulla, where they are co-stored with amines and peptides. The members of this protein class are named chromogranin A (CgA), chromogranin B (CgB), and secretogranin II (Sg II), each showing a widespread neuroendocrine expression (Hutner et al., 1991; Winkler and Fischer-Colbrie, 1992; Taupenot et al., 2003). Concerning the endocrine pancreas, chromogranins were described as typical components of the endocrine cell types in several mammals (Rindi et al., 1986; Grube et al., 1986). However, in some cases, findings were controversial according to the particular mammalian species and the antisera used. In the endocrine pancreas of rats, CgA positive B-cells could only be demonstrated using an antiserum raised against rat CgA (Grube et al., 1989), whereas in A-cells, a faint staining was found when an antiserum raised against bovine CgA was applied (Grube et al., 1986). Immunoreactivities for CgB were generally to be found of low intensity compared with CgA, while Sg II-immunoreactivities have not previously been described for rat B-cells. Concerning our studies on RINm5F cells, immunoreactivities for CgA and CgB were expressed reversely compared with normal B-cells. CgB was found most frequently as an intragranular compo-
nent and thus could be regarded as a "marker protein" for this insulinoma cell line, while CgA was found only in a minority of RINm5F cells. Data on the occurrence and function of chromogranins and related peptides in RINm5F cells are rare. Swarowsky et al. (1994) showed that sodium butyrate increased the CgA mRNA levels in RINm5F cells. A complete absence of CgA expression in RINm5F cells was described by Kayo et al. (1996), who, however, found it in the MIN6 cell line. As for the occurrence of Sg II in the endocrine pancreas, immunoreactivities were only found in bovine PP-cells (Yoshie et al., 1987). In RINm5F cells, SG II positive cells were very rarely detected in the present study. This is in accordance with findings by Sher et al. (1995) on normal untreated RINm5F cells. These authors, however, demonstrated an increase in SG II as well as insulin expression after NGF treatment.

Various biological functions of chromogranins have been proposed, such as the binding of amines or calcium, involvement in granulogenesis, the sorting or packaging of peptide hormones or their precursors, or possible regulatory functions by derived peptides (for review see Taupenot et al., 2003; Feldmann and Eiden, 2003). Of the chromogranins, CgB was found to play a regulatory role in islet IAPP and insulin secretion, obviously by an autocrine inhibitory effect (Karlsson et al., 2000). The chromogranin-derived peptide pancreastatin, originally isolated from the porcine pancreas (Tatemoto et al., 1986), was found colocalized with CgA in a minority of RINm5F cells. The inhibitory effect of pancreastatin on insulin secretion has been reported in the rat pancreas (for review see Sanches-Margalef et al., 1996) and in RINm5F cells (Hertelendy et al., 1996). Furthermore, pancreastatin has been found to increase cytosolic Ca^{2+} levels (Sanches-Margalef et al., 1992).

Serotonin, amine transporter, and PP-related hormones

In the pancreas of various vertebrates enterochromaffin (EC-) cells, islet cells or nerve fibers have been found to represent serotonin-containing structures (Ding et al., 1991). While islet B-cells in mice were identified as storing serotonin (Hellman et al., 1972), in rats serotonin was demonstrated mainly in the neural elements of the pancreas (Adeghate et al., 1999). In RINm5F cells, however, we could clearly show serotonin immunoreactivities in a subpopulation of cells. The serotonin content of these cells often showed a negative correlation with the insulin content. Our immunohistochemical proof of serotonin positive RINm5F cells is in accordance with previous biochemical assays indicating that RINm5F cells can synthesize, take up, and release serotonin (Richmond et al., 1996). The physiological relevance of serotonin for insulin release is still undetermined. Serotonin, however, does not seem to be an important regulator of insulin release because high concentrations of serotonin are needed to influence glucose-induced insulin release (Lindstrom and Lundquist, 1994).

In the present study we have demonstrated the expression of VMAT1, one of the two isoforms of vesicular monoamine transporters involved in the accumulation of monoamines in the secretory granules (Henry et al., 1998). Compared with immunoreactivities for serotonin, those for VMAT1 were found in a higher number of RINm5F cells, suggesting that VMAT1 can be expressed regardless of their amine content. During the embryonic development of rats, VMAT1 was detected in diverse organs of the endocrine system (Hansson et al., 1998). In adult rats VMAT1 expression was found exclusively in endocrine cells of the adrenal medulla, intestine, and stomach (Weihe et al., 1994; Erickson et al., 1996). Recent studies on VMAT1 expression in human gastrointestinal endocrine tumors have demonstrated the expression of VMAT1 in insulinoma cells (Jakobsen et al., 2001) and in serotonin-containing pancreatic endocrine tumors (Anlauf et al., 2003). These findings correspond to our observation on VMAT1 expression in rat insulinoma cells.

With respect to the hormones of the PP family, we found NPY and PYY but no PP immunoreactivities in RINm5F cells. Our findings for the NPY expression do not support those of Myrsen-Axcrona et al. (1997), who found no NPY expression in RINm5F cells under control conditions but after treatment with dexamethasone. This discrepancy may be due to differences in the specificity of the NPY antisera or the number of passages of cell culture. NPY secretion has also been shown from human insulinoma cells when kept in a primary cell culture (Wieber et al., 1996). In the normal rat pancreas, however, NPY is confined to neuronal elements (Sundler et al., 1993) but can be expressed by B-cells after dexamethasone-treatment (Myrsen et al., 1996).

The cellular localization of PYY, which we observed in only a few RINm5F cells and exclusively in cells lacking insulin content, shows some species variation in normal pancreatic islets. Thus, PYY coexists with glucagon in rats, whereas in other species it seems to be colocalized with PP (Sundler et al., 1993).

In conclusion, the heterogeneity of the insulinoma cell line RINm5F was elucidated in a morphological and immunohistochemical analysis of semithin and ultrathin sections. The present study demonstrates that RINm5F cells differ in ultrastructural aspects, mainly in the number and appearance of their secretory granules as well as in their immunoreactivities for insulin, serotonin, and further peptides and proteins. The colocalizations of these immunore-
activities exhibited quite different phenotypes of RINm5F cells. These peculiarities may reflect different functional states or phases of cytodifferentiation in this insulin-secreting cell line.

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