The Ultrastructure of Polyploid B-Cells in the Islets of Normal Mice

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Summary. Light and electron microscopic studies of diploid, tetraploid and octaploid B-cells in the islets of normal C57BL/KsJ mice revealed that polyploid cells were characterized by a wider range of granulated states than diploid B-cells. The maximum granule densities were similar for polyploid and diploid cells; however, some polyploid cells were almost devoid of granules, while the least granulated diploid cells contained intermediate granule densities. The tetraploid cells also appeared to be characterized by an increased mitochondrial stage which suggests compensation for the greater degree of degranulation. These observations were confirmed by morphometric analysis. Two interpretations of the apparent polyploidy are discussed; that polyploid B-cells may be more responsive to insulin releasing stimuli than diploid B-cells and that tetraploid cells may only be diploid cells in the G_2 phase of the mitotic cycle.

Key words: Pancreatic islets in mice, genotype: C 57BL/KsJ, ultrastructure, B-cells, nuclear volume classes, polyploidy, DNA replication, morphometric analysis.

Despite suggestions that polyploid cells are functionally differentiated from their diploid counterparts and may be associated with increased synthetic activity or other active metabolic processes [1–4], the precise functional significance of the polyploid state remains obscure. A major problem has been the identification of a polyploid cell system of known function and constant nuclear DNA variation.

With the discovery and characterization of polyploid B-cells in human pancreatic islets by Ehrie and Swartz in 1974, a polyploid system in a cell type of

well-defined function was established [5]. In further studies, Ehrie and Swartz have indicated that polyploid B-cells are also characteristic of islets of normal C57BL/KsJ and mutant diabetic C57BL/Ks-db/db mice [6]. They concluded that normal human pancreas is characterized by three DNA states, diploid ($2n$), tetraploid ($4n$) and octaploid ($8n$), and that B-cell nuclei in twelve week C57BL/KsJ and C57BL/Ks-db/db mice also exhibit distinct DNA-volume classes. In addition, they concluded that the nuclear DNA content of the islet B-cell was directly proportional to the volume of its nucleus. In part, these conclusions form the basis for this paper.

The present work was carried out in an attempt to determine whether any significant ultrastructural differences might exist between B-cells with diploid and polyploid nuclei in the islets of Langerhans of normal C57BL/KsJ mice, differences that might suggest functional differentiation with polyploidy.

Materials and Methods

Animals and Diet

Three C57BL/KsJ male littermate mice were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and allowed free access to Purina Laboratory Chow and water. At twelve weeks of age, tail lengths and body weights were recorded and the animals killed by cervical dislocation.

Preparation for Light and Electron Microscopy

The pancreata were excised, minced into 1 mm³ blocks and fixed in 0.1 M phosphate buffered 4% glutaraldehyde solution (pH = 7.3) for one and

Fig. 1. Maximum mean nuclear volume determinations of pancreatic islet cell nuclei from serial semi-thin and serial thin and adjacent semi-thin sections of islets from three normal 12 week C57BL/KsJ mice

one-half hours [7]. The blocks were then placed in 0.1 M phosphate buffer (pH = 7.3) for fifteen minutes and subsequently postfixed in 0.1 M phosphate buffered 1% OsO₄ (pH = 7.3) for two hours [8]. Routine procedures for embedment in Epon 812 [9] were then followed.

Light Microscopy

One block was selected at random from each of the three mice and semi-thin Epon sections (1.0–1.5 μ) were cut on a Porter Blum MT-2 ultramicrotome using a diamond knife. The semi-thin sections were placed on glass slides and islet tissue was identified by staining with hematoxylin and eosin [10], Munger's modification of the aldehyde thionine hematoxylin eosin stain for pancreatic islets embedded in plastic [11], or toluidine blue [12]. Toluidine blue staining proved to be a simple and reliable method for differentiation of islet and acinar tissue, and was employed as the stain of choice for further studies with light microscopy.

Once an islet was identified, serial semi-thin sections were cut. The sections were stained with toluidine blue and subsequently used for nuclear volume class determination. Light micrographs were taken using a high dry apochromatic objective lens (40:1, N.A. = 0.95).

Electron Microscopy

To permit correlation of nuclear volumes of cells with their location within an islet, sixteen gray to silver

sections were cut, followed immediately by a semi-thin section. Six to eight sections were then cut to clear the block face and the process was repeated. The serial thin sections were mounted on 75 mesh copper grids coated with a 0.6% parlodian solution film, stained with uranyl acetate [13] and lead citrate [14] or lead hydroxide [15] and viewed in a Philips 200 electron microscope. The magnifications of each twelve plate cassette were referenced using a Fullam grid with 54,864 lines per inch.

Determination of Nuclear Volume Classes and of Individual Nuclear Volumes

Since Ehrie and Swartz determined that, when the B-cell nuclei in the C57BL/KsJ mice are treated as spheres, their volumes are directly proportional to their DNA content [6], nuclear volume classes in this study were determined in the same manner. The mean orthogonal diameters of B-cell nuclei in the serial semi-thin, toluidine blue stained, Epon sections were measured with a calibrated filar micrometer. All nuclei in each section were referenced with regard to their position in the islet and the mean orthogonal diameters of all sections through the same nucleus were ordered. The maximum mean diameter was then used to calculate its nuclear volume. All semi-thin section nuclear volumes were determined using an oil immersion lens (90:1, N.A. = 1.32).

For ultrastructural analysis, the mean diameters of the nuclei in contiguous thin sections were ordered with the diameters from the adjacent semi-thin sections. The maximum mean diameter in the ordered set was used to calculate the nuclear volume.

Stereological Methods

Utilizing the point count technique of Weibel [16], the beta granule and mitochondrial volume densities (V_v) were calculated in two of the three most degranulated diploid cells seen, and in eight diploid and fourteen tetraploid cells selected at random. The average values resulting from morphometric analysis of three to five sections, from each of seven tetraploid cells, indicated that a single section analysis provided granule and mitochondrial volume densities within 2.8 volume per cent of the calculated mean values. The significance between the granule volume densities of the diploid and tetraploid B-cells was determined by the nonparametric Mann-Whitney "U" test [17]. Since insufficient numbers of tetraploids were available, it was not possible to determine if the two tetraploid cells with markedly increased mitochondrial densities (Fig. 5) were statistically significantly different from the remaining cells on the curve.

Fig. 2. Section of a pancreatic islet containing a poorly granulated tetraploid B-cell which borders the external islet surface and a capillary (arrow). Toluidine blue

Results

Animals

The body weights (24.2–26.3 gms) and tail lengths (8.1–8.4 cms) of the three 12 week C57BL/KsJ mice used in our study compare favorably with those reported previously [6] for C57BL/KsJ mice which exhibited distinct B-cell DNA volume classes.

Nuclear Volume Classes

The distribution of nuclear volumes calculated from the maximum mean diameters of nuclei in the semi-thin series, as well as from the adjacent thin and semi-thin sections, are presented in Figure 1. The mean nuclear volumes for each of the three classes are presented in the upper right hand corner of Figure 1. Nineteen tetraploid and three octaploid nuclei were found in five of the thirteen islets studied. The distribution of polyploid cells in the five islets is shown in Table 1. Tetraploid and octaploid B-cells accounted for 5.2% and 0.8%, respectively, of the total number of B-cells studied in these five islets. The maximum

mean diameters of all nuclei studied in the thirteen islets gave nuclear volumes which fell within the limits of each of the three volume classes.

Light Microscopy

Light microscopic examination of semi-thin sections revealed considerable variation in islet size, shape and staining characteristics. The mean diameters of the islets ranged from 45 μ to 250 μ , while the shapes varied from essentially spherical to prolate spheroids. Most of the islets contained cells with differing degrees of stain density, but a few exhibited a predominantly moderate or dark staining appearance. Figure 2 shows an islet which is characterized by cells of varying stain densities and which contains a lightly staining tetraploid B-cell. Figure 3 illustrates a moderately staining islet which contains two octaploid B-cells. Polyploid cells were found in some moderately and mixed staining islets, but not in uniformly dark staining islets. At the ultrastructural level, the lighter staining cells were seen to reflect a less granulated state than the darker staining cells. In general, polyploid B-cells were located on the external islet surface or

Fig. 3. Section of a pancreatic islet containing two octaploid B-cells; one octaploid (1) borders a capillary not seen in this section; the second octaploid (2) lies adjacent to the external islet surface. Toluidine blue

Table 1. Distribution of diploid, tetraploid and octaploid B-cells in the five islets which contained polyploid B-cells

Islet	Diploid	Tetraploid	Octaploid	% Polyploid
A	35	2	0	5.4
B	63	2	0	3.2
C	72	2	0	2.7
D	55	0	2	3.5
E	116	13	1	10.8
	341	19	3	Mean = 6.0%

adjacent to islet capillaries, while diploid cells were more centrally located in the islet and exhibited no particular spatial relationship to islet capillaries.

Electron Microscopy

The adjacent thin and semi-thin sections permitted correlation between cell types, nuclear volumes and the location of individual cells within an islet.

1. Diploid B-Cells. A study of the diploid cell fine structure revealed differences in granule content (Fig. 4) which were reflected in the stain density using light microscopy (Fig. 4, inset). Morphometric analysis of the diploid cells indicated a minimum granule volume density of 11.2%, a maximum of 30.5% and a gradation of granule densities between these values (Fig. 5). Most of the diploid cells in the thirteen islets were characterized by granule densities in the upper range.

Ultrastructurally, the mitochondria in diploid cells appeared similar in form, shape and density throughout the range of granulated states; however, morphometric analysis revealed some variation with a

moderate increase, of questionable significance, in mitochondrial density of cells with a granule density near 17% (Fig. 5).

A correlation between granule content, cell matrix density and type of ER was seen within the range of diploid granulation. The more degranulated diploid cells were characterized by vesicular or saccular rER and a lighter electron density of the cellular matrix, while the more granulated cells contained lamellar rER and increased electron densities of the cellular matrix (Fig. 4).

2. Tetraploid B-Cells. Although the nineteen tetraploid cells represent only a small percentage of the total number of cells studied, their fine structure revealed a greater range of variation than that seen in the diploid cells. The most obvious differences were a greater degree of degranulation and a stage of increased mitochondrial content.

Fourteen tetraploids were analyzed morphometrically with regard to their granule and mitochondrial volume densities (Fig. 5). The data revealed a minimal granule density of 0.5% and a gradation of granule densities up to a maximum of 30.6%. Six of the fourteen tetraploids exhibited granule densities less than the least granulated of the diploid B-cells. Figure 6 illustrates one example of a degranulated tetraploid B-cell. Morphometric analysis of five sections from this cell indicated an average granule volume density of 3.7% and an average mitochondrial volume density of 4.4%. The cytoplasm contained small numbers of distinct beta granules, many vesicles studded with ribosomes and compact groups of microvesicles. The cytoplasmic content of vesicular rER was most pronounced in tetraploid cells with low granule densities;

Fig. 4. An electron micrographic collage of a portion of a pancreatic islet which illustrates the range of granulated states characteristic of diploid B-cells. A = A-cell, C = capillary. Inset: adjacent semi-thin section showing the stain densities, which reflect granule content, and the location of the cells within the islet. Toluidine blue

Fig. 5. Granule and mitochondrial volume densities (%) of diploid and tetraploid B-cells. Each point represents one cell

as the granule content increased, the vesicular rER component decreased and was eventually replaced, at higher granule densities, by lamellar rER.

In observing the number and shape of mitochondria as a function of granule content, an interesting phenomenon was noted. The mitochondrial density in tetraploid B-cells appeared relatively constant; however, a marked increase was seen between granule densities of 11% and 14% (Fig. 5). Two of the nineteen tetraploid cells, both containing granule densities near 12%, were characterized by extensive networks of mitochondria which appeared in a variety of elongate, branched or curved forms. No diploid cells containing as distinctive a cytoplasmic mitochondrial component were ever seen. Figure 7 illustrates the appearance of the mitochondria in one of these cells. The average granule and mitochondrial densities ($n = 3$) of this cell were 12.4% and 16.9%, respectively. The beta granule content was increased considerably over that in Figure 6 and free ribosomes were seen in the cytoplasm. A close relationship of this cell to both a capillary and the external islet surface was seen in the adjacent semi-thin section (Fig. 7, inset).

As the granule content increased beyond the stage of increased mitochondrial density, the fine structural

characteristics of the tetraploid cells merged with the ultrastructural characteristics of the diploid cells. Except for distinct nuclear volumes, the more highly granulated diploid and tetraploid cells could not be differentiated.

Like the diploid B-cells, a correlation between granule content, type of ER and cellular electron density was apparent in the tetraploid B-cells.

3. Octaploid B-Cells. Due to the limited number of octaploid B-cells encountered, no attempt was made to order them according to their granule and mitochondrial content. However, all three octaploid cells could be placed within the limits of the granule densities characteristic of the tetraploid cells. The two octaploid cells in Figure 3 were ultrastructurally indistinguishable from the surrounding diploid cells characterized by similar granule densities. The third octaploid cell was characterized by margined beta granules, vesicular rER, increased mitochondrial branching and increased cellular length (approx. 33 μ). On the basis of its ultrastructure, the third octaploid cell was comparable to the more degranulated tetraploid B-cells.

Discussion

The B-cell nuclear volume classes presented in Figure 1 exhibited ratios closely approximating 1:2:4, and correspond to the nuclear volume class ratios reported by Ehrie and Swartz in their Feulgen microdensitometric studies of 12 week C57BL/KsJ and C57BL/Ks-db/db mice [6]. The 17%, 19% and 21% increases in mean nuclear diameter over those previously reported most likely indicate a difference between islets fixed with Zenker-formol and embedded in paraffin and those fixed with glutaraldehyde-OsO₄ and embedded in Epon 812. In general, these consequences of fixation at the light and electron microscopic levels are well known. The consistency of the increase in mean nuclear diameters and the similarity of the nuclear volume distribution curves, indicate that the volume classes in this study reflect accurately the diploid, tetraploid and octaploid values of DNA characteristic of the volume classes reported by Ehrie and Swartz.

As indicated in the results section, a correlation between electron density and form of endoplasmic reticulum with granule density was found. These may represent artifacts of fixation since previous EM studies on pancreatic islet tissue from C57BL/KsJ mice fixed by perfusion revealed no correlation between these characters and the granule content [18].

Although the relatively small number of polyploid cells studied may not reflect accurately the full range

Fig. 6. Portion of a poorly granulated tetraploid B-cell which contains numerous profiles of mitochondria, free cytoplasmic ribosomes and intranuclear filaments (pointer)

or degree of cellular variation characteristic of polyploid cells, it is nevertheless evident that they exhibit a greater range of granulated states than do diploid B-cells. Notably, this is due, not to differences in maximal granule volume densities, but to significantly lower granule densities among the tetraploid cells ($Z < 0.02$). The presence of two tetraploid cells exhibiting a dramatically increased mitochondrial compartment (Fig. 7) and containing similar granule densities (Fig. 5) suggests, but does not prove, that the greater degree of tetraploid degranulation may be compensated for by an increased potential for energy production related to granule synthesis. Clearly,

further studies focussing on tetraploid granule densities approximating 12% are necessary before an increased mitochondrial stage in tetraploid cells can be confirmed.

The observations that tetraploid cells usually border either the external islet surface or islet capillaries and, ultrastructurally, appear to be normal, active cells, suggest that polyploid cells constitute a cell line which is apparently more responsive to normal stimulation than diploid cells, having the capacity to degranulate completely under conditions which induce only partial degranulation of diploid B-cells. This interpretation is in agreement with other studies

Fig. 7. Micrograph of a partially granulated tetraploid B-cell which contains numerous mitochondria, free cytoplasmic ribosomes and intranuclear filaments (pointer). Inset: Adjacent semi-thin section. The tetraploid cell (arrow) borders the external islet surface and a capillary. Toluidine blue

on localized tissue polyploidy, which suggest that polyploid cells are associated with increased efficiency and metabolic processes [19–21]. If this interpretation is correct, a morphologic basis may exist for the well-documented biphasic release of insulin [22–27]. The existence of small numbers of apparently more responsive polyploid cells in close proximity to islet capillaries suggests the possibility that the first phase of

insulin release may reflect, in part, the differential release of insulin from polyploid B-cells, while the second phase reflects the slower release of insulin from larger numbers of diploid cells.

It is interesting to note that Boquist et al., in studying C57BL/KsJ-db/db and C57BL/6J-db/db diabetic mice, described hypertrophic B-cells characterized by large nuclei, prominent Golgi complex and endoplas-

mic reticulum. The authors suggest that these cells "may reflect a compensatory hyperfunction of the endocrine pancreas in response to hyperglycemia of unknown genesis" [28].

Although we have assumed that the DNA-volume classes reflect a true polyploidy of B-cells, an alternative explanation may be based on studies showing that some DNA replication occurs under normal physiological conditions [29–33], and that at least some of the DNA replication reflects preparation for mitotic division [34–37]. The interpretation that DNA replication in B-cells represents only preparation for cell division, suggests the possibility that cells with multiple DNA classes may only be diploids in the G_2 phase of their mitotic cycle.

There are, however, several problems inherent in this interpretation. First, if it is assumed that "tetraploid" cells are merely diploid cells in G_2 , it becomes difficult to explain the "octaploid" B-cells seen in this study and in others [5, 6]. On this basis, an "octaploid" cell must be the G_2 stage of a non-existent "tetraploid" cell. Secondly, we are unaware of studies that would support the existence of as wide a range of cytological variation during G_2 as have been observed among the tetraploid cells in this study. Furthermore, Like and Chick have observed that "there are very few labelled cells among the islets of normal mice (C57BL/KsJ) at all ages (4–28 weeks) examined. In most instances a random section of the entire pancreas contains a total of not more than 5 labelled (H^3 -thymidine) islet cells" [38]. These observations are of interest because our preliminary studies indicate that 4 week C57BL/KsJ mice contain fewer, and 16 week mice more, polyploid cells than 12 week mice (R. E. Bowen and F. J. Swartz, unpublished results). This suggests that the mitotic rate may not correlate with increased polyploidy. It is also interesting to note that in an ultrastructural study of monolayer islet cultures incubated at high glucose concentrations (16.5 mM), three classes of epithelioid cells containing small, medium and large nuclei were described [37]. The size of the nuclei correlated inversely with the degree of aldehyde-thionine staining. If it can be assumed that the three nuclear classes observed correspond to the three polyploid classes reported in these studies, and that the degree of aldehyde-thionine staining is related to the granule volume density, then the increased amount of insulin released into the culture medium in response to high glucose levels would be consistent with the interpretation that polyploid B-cells are more responsive to stimulation than are diploid cells. Lastly, the close anatomical and embryological relationship of the pancreas to the liver, in which the existence and development of polyploidy has been documented [39], is further indirect support

for the interpretation that true polyploidy may exist in pancreatic islets.

Clearly, the problem of whether some B-cells are polyploid cells characterized by a greater response to normal stimulation or diploid cells in G_2 remains to be resolved. However, until chromosome counts are made and the mitotic indices can be correlated with numbers of polyploid cells, results derived from B-cell DNA labelling studies should be interpreted, at least in part, as a reflection of polyploidization. The observation that tetraploid and octaploid cells can be less granulated than the most degranulated diploid cells, under identical physiological conditions, suggests that at least some polyploid cells may be more responsive to insulin release stimuli than diploid B-cells.

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