Submicroscopic Structure of Yoshida Ascites Hepatoma*

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SUMMARY

The electron microscope study of Yoshida ascites hepatoma cells confirms their epithelial origin. Isolated cells may occur, but they are mostly gathered to form islets. Cell surfaces exhibit microvilli. Cells are interconnected by means of microvilli and of structures similar to the epithelial "terminal bars." The cells line spaces and canaliculi similar to bile canaliculi. The cytoplasm is poorly differentiated; membrane systems and intracellular organelles are poorly developed, with a relative increase of Palade granules. The Golgi complex is well developed. The cytoplasm also contains "fibrillar formations," lipide and granular inclusions. There are frequent vesicles of variable size. Virus-like particles have never been observed. Involution and degeneration of cellular components are frequently found in Yoshida ascites hepatoma cells.

Electron microscopy has been largely used in these last years to investigate the submicroscopic structure of tumor cells (3). Various spontaneous or transplantable tumors have been examined, including some classical ascites tumors, such as the Ehrlich ascites carcinoma of the mouse (1, 3, 5, 17, 18, 21-23) and the Yoshida sarcoma of the rat (17, 21, 23).

Yoshida, Sato, and Aruji (25) produced an ascitic form of hepatoma by injecting into the peritoneal cavity of the rat a cellular suspension obtained by macerating a dimethylaminoazobenzol-induced solid hepatoma. They formerly described the cytological characteristics of the ascites hepatoma. Sato, Essner, and Belkin (14, 15) perfected the separation technic using enzymes, being able to follow the development of the tumor from isolated cells to the formation of cell islets.

The aim of the present research has been to study with the electron microscope the submicroscopic structure of the rat Yoshida ascites hepatoma, a tumor which is being investigated in this laboratory from the biochemical point of view.

MATERIALS AND METHODS

The Yoshida hepatoma AH 130 (Yoshida) has been maintained in albino rats since 1958, when the tumor was obtained from the Laboratori Farmitalia, Milano, to which it was supplied by the Institute of General Pathology, University of Milan, Via Mangiagalli 31, Milan, Italy.

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Medical Institute of the Sasaki Foundation, Tokyo, Japan. The ascitic fluid was removed with a syringe from rats inoculated 6-7 days earlier. An aliquot (1.0 ml.) of fluid was immediately mixed with 5 volumes of Palade fixing fluid; a second aliquot was centrifuged at 1000 X g for 2 minutes at room temperature to sediment tumor cells. In the latter case the sedimented cells were washed once in a volume of a modified Krebs-Ringer buffer (12) equal to twice the original volume of the ascitic fluid. The washed cells deriving from 1 ml. of ascitic fluid were than treated with 5 ml. of the aforementioned fixative. Fixation was carried out at 1°-2° C. for 30 minutes. The fixed cells were then separated by centrifugation, dehydrated rapidly in a series of graded ethyl alcohols, and embedded in a mixture (8:2) of n-butyl and methyl-methacrylate containing 1.5 per cent of benzoyl-peroxide as a catalyst. Some of the methacrylates were previously polymerized. Polymerization of methacrylate was obtained in the oven at 54° C. for 18 hr., followed by a 3-day stand at room temperature. Some samples were embedded in Araldite (6). Sections (25-60 μ) were cut with a Servall Porter-Blum microtome, using glass knives. The sections were mounted on grids coated with formvar or on carbon films and examined with the Siemens Elmiskop I or Elmiskop II microscopes. Sections of the material embedded in methacrylates were stained with uranyl acetate (20), whereas the epoxide-embedded tissue was stained with uranyl acetate or with potassium permanganate (8).
Phase contrast microscopy was routinely used to examine either fresh cells in suspension or sections of 0.5–1 μ of the material embedded in methacrylates. Observations were also made on smears stained with Feulgen reagent and light-green as a counterstain.

RESULTS

LIGHT MICROSCOPY

Microscopic observations, made either on smears stained with Feulgen reagent or on fresh cells examined by phase contrast microscopy, substantially confirmed the findings by Yoshida et al. (24, 25). Hepatoma cells may appear isolated, but more frequently they form “pairs” or islets. Cells have a variable size, and those of islets are polyhedral in shape. Nuclei appear rather irregular. Mitotic figures are frequent, and in a number of cases they are abnormal. The cytoplasm is often vacuolated; when cells form “pairs” or islets, large vesicles may be seen in the contact areas. Phase-contrast microscopy clearly reveals strongly refractive lipide inclusions. The so-called “crescent-shaped cells” described by Sato, Essner, and Belkin (14, 15) were never observed in our preparations, and it was found almost impossible to make a distinction between epithelial and endothelial cells. It will be shown on what even the submicroscopic characteristics of ascites hepatoma cells support their epithelial origin.

ELECTRON MICROSCOPY

A general finding which emerges from the examination of a large number of sections is the great variability in the submicroscopic structure of the cells. Furthermore, several cells show serious degenerative changes in their structure. For these reasons the present results limit themselves to the description of well preserved cells, whose characteristics seem to be significantly like those of most of the cells examined.

FORM AND RECIPROCAL RELATION OF THE CELLS

The cells may be either isolated (Fig. 1) or assembled in small groups (Figs. 2, 3) of three or four elements, from which they pass gradually to masses which may reach a macroscopic size. There is a minimum percentage of isolated or paired cells which are always spherical in form and show on their surfaces numerous digitations which have the typical aspect of “microvilli” (Figs. 1–3). Cells assembled into islets assume a polyhedral form, and they may then reach a compact arrangement as in the epithelial tissue (Figs. 2, 3). The lines of contact between the cells are irregular, and very often the limiting membranes are separated by microspaces. These intercellular microspaces show a great variation in both size and shape (Figs. 2, 3). Occasionally, they are simple canals similar to bile canaliculi; sometimes they consist of large dilatations which may join up to form a vesicle lined by a thin layer of cells. Microvilli and cell membrane processes can often be found projecting into spaces, canals, and dilatations (Figs. 2, 3, 7).

When in close contact, cell membranes appear thickened and more opaque to the electron beam (Figs. 2–5). Along these thickened membranes, particularly when the sections were “stained” with uranyl acetate, a gathering of fine filaments (30–50 A) may be observed (Fig. 4). This picture closely resembles the aspect of the so-called “terminal bars” of the epithelia. Occasionally structures which can be clearly classified as “desmosomes” are seen (Fig. 6). Assuming the existence of a cementing substance as in epithelial tissues, it is reasonable to suppose that the cementing substance which binds the hepatoma cells together (14) is localized at these points. Electron microscopy did not reveal any morphological equivalent of the cementing substance. However, in sections cut perpendicularly to the cell membrane, a space about 100 A thick and possessing low and homogeneous electron absorption may be observed between the membranes of adjacent cells (Fig. 5).

STRUCTURE OF THE CELLS

a) Nucleus.—The nuclei are irregular, frequently lobated, with deep infolding (Figs. 2, 3); the nuclear envelope, made of two sheets, is about 300 A thick. The nuclear content is in the form of homogeneously dispersed granules, but it may appear aggregated into a coarse meshwork which is more dense in proximity to the nuclear envelope. Nucleoli are voluminous, very irregular, and of reticular appearance. The strands are formed by packed granules and delimit wide spaces having low electron absorption (Figs. 1, 2, 3).

b) Cytoplasm.—One can usually distinguish in the cytoplasm the limiting membrane, the fundamental part or “hyaloplasm,” and the endocellular organelles. In addition, some peculiar formations may be visible, such as the so-called cytoplasmic inclusions, which are formed by spheroidal bodies of granular type, lipide droplets, and filamentous structures.

LIMITING MEMBRANE

The limiting membrane usually appears as a thin osmiophilic layer (80 A) deeply stained by uranyl acetate; it is generally rather irregular, and even with the higher magnification it is impossible to distinguish its component layers as in other types of cells (18).
HYALOPLASM

There appear to be two main types of hyaloplasm structure. One of these is characterized by the prevalence of a low, dense matrix with a marked concentration of Palade granules (9) (Figs. 8, 11). Vesicles delimited by a thin membrane with or without associated Palade granules are the main feature of the other type; this typical electron microscope image of the osmium-fixed hyaloplasm corresponds to the so-called endoplasmic reticulum (10) (Figs. 7, 9).

Between these two extremes there are intermediate aspects. The fact that poorly differentiated cells with scarce membrane systems contain a high concentration of free granules in the hyaloplasm indicates that a direct relationship does not exist between the development of membranes and the amount of Palade granules. After "staining" with uranyl acetate all hyaloplasmic structures stand out more clearly and appear thickened. A delicate reticulum of filaments (30–50 Å) is visible among vesicles and groups of Palade granules (Fig. 11). This fine hyaloplasmic reticulum has also been demonstrated in normal cells (2,11).

INTRACELLULAR ORGANELLES

Intracellular organelles, i.e., mitochondria and ergastoplasmic systems, are poorly developed. Experiments still under investigation clearly show that ascites hepatoma cells, as compared with normal liver cells, have a reduced number of mitochondria and a poorer development of their total surface. The structure of the mitochondrion appears typical (19); there is a double outer membrane, an inner system of membranes, and homogeneous contents. Mitochondria are mainly oval and occasionally rod-shaped. The arrangement of the inner membranes is variable; occasionally they are continuous either across the mitochondrial cavity (Fig. 13) or parallel to the longer axis of the mitochondrion (Fig. 14); sometimes they appear curved (Fig. 15).

The arrangement of the inner membranes differs from that present in normal liver mitochondria, where it seems more accurate to speak of "cristae" rather than of membranes, since they rarely cross the whole mitochondrion. Frequently mitochondria appear swollen and vacuolated (Figs. 16–18).

Typical ergastoplasm with double membranes rich in Palade granules are not visible. Occasionally one or two rows of membranes are found; usually, however, one find rows of oval and spherical vesicles with associated Palade granules (Fig. 9). Occasionally one may see structures classified as "membrane fenestratae" or "annualate lamellae" (16) (Fig. 12).

The Golgi complex is very well developed (Figs. 1, 7, 10). It consists mainly of vesicles and microvesicles, with few parallel membrane systems and large vesicles. Sometimes one may find more than one Golgi complex in the same section of the cell.

Smooth vesicles of variable size are found scattered in the cytoplasm (Figs. 1, 7); they appear larger at the periphery of the cell.

In cells in which cytoplasmic structures appear more developed, areas of filamentous structures may be found which occur in a compact arrangement either in straight formations or in whorls. They are roughly 80–100 Å in diameter and probably consist of microglobular chains (Figs. 20, 21).

These filamentous areas are found close to the nuclei, sometimes between their lobes. Often the bundles of fibrils surround lipid droplets or the Golgi complex (Figs. 3, 20).

The fibrillar structures are similar to the so-called "fibrillar formations" demonstrated by Freeman and Samuel (4) in the cytoplasm of human leukemic leukocytes.

Lipide inclusions in the form of irregularly outlined droplets are numerous and large (Figs. 2, 3, 10). They are often near the Golgi complex or among the bundles in the filamentous areas.

Granular inclusions are also seen. They appear as round, very dense bodies (Fig. 22). These inclusions occasionally exhibit a central cavity and walls which appear to be composed of a lamellar system (Figs. 3, 7, 22, 23).

DISCUSSION

The study of the submicroscopic structures of the Yoshida ascites hepatoma cells has confirmed the data obtained by light microscopy and has stressed the great variability in size and shape of all cellular elements. In addition, the results of the present study allow some interesting considerations.

The submicroscopic structures of the Yoshida hepatoma cells appear different from that of normal liver cells. The diversity refers mainly to the development and structure of mitochondria and ergastoplasmic systems.

Yoshida ascites hepatoma cells can be easily differentiated from those of other ascites tumors. Thus, while cells of Ehrlich ascites tumor or Yoshida ascites sarcoma are isolated units, ascites hepatoma cells are typically arranged as described above. Furthermore, virus-like particles have been frequently demonstrated in Ehrlich tumor cells (1, 3, 5, 17, 18, 21–23), but they were never seen.
in cells of the ascites hepatoma. The only diagnostic doubt might arise from a submicroscopic comparison of isolated elements of hepatoma and Yoshida sarcoma. The isolated cells actually appear very similar. It is of interest to recall here that the submicroscopic structure of solid hepatoma cells (Novikoff tumor) as described by Howatson (7) is comparable to the structure of ascites hepatoma cells.

The above results and observations point clearly to the fact that, even in different tumors, cells share common submicroscopic characteristics—that is, undifferentiated cytoplasm, poor development of intracellular organelles, hypertrophy of the Golgi complex, frequent degenerative changes, and the presence of cytoplasmic inclusions (3). The characteristics of this undifferentiation are shared also by some normal cells such as embryonic cells, seminal cells, cells of the germinal layers of the epidermis, and the rapidly growing cells of lymphatic tissues.

The main characteristic of Yoshida ascites hepatoma cells is that, although generally undifferentiated, they retain some organotypical properties. Thus, the contact areas between the cells such as “terminal bars” or “desmosomes” show an innate tendency to cell association and to tissue formation. Since these structures and properties have been demonstrated so far in epithelia, the epithelial origin of ascites hepatoma cells seems likely. Moreover, the presence of canaliculi and intercellular spaces with microvilli, the hypertrophy of the Golgi complex, and the demonstration of cytoplasmic vesicles may be related to secretory processes and may be interpreted as signs of some differentiation.

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REFERENCES

The mark in each figure represents 1 μ. All photographs, unless otherwise specified, refer to methacrylate-embedded tissue.

Fig. 1.—Yoshida ascites hepatoma cells; isolated elements. 

mv = microvilli; m = mitochondria; g = Golgi complex; n = nuclei; nl = nucleoli. Mag. ×9,000.

Fig. 2.—Yoshida ascites hepatoma cells, portion of large “islet.” The arrows show the contact points between the cells. 

l = lipide inclusions; c = canals and intercellular spaces. Mag. ×4,000.
Fig. 3.—Yoshida ascites hepatoma: section of small “islet.” The arrows show the contact point between the cells. = intercellular spaces; f = fibrillar formations; g = Golgi complex; i = granular inclusions; m = mitochondria; l = lipide inclusions; nl = nucleoli. Mag. ×6,500.
Fig. 4.—Portion of Yoshida ascites hepatoma cell; contact points (terminal bars) with a gathering of filaments along the cell membranes. $m =$ mitochondria. This section was “stained” with uranyl acetate. Mag. $\times$33,000.

Fig. 5.—Contact points between the cells. Section of “Araldite”-embedded tissue “stained” with potassium permanganate. Mag. $\times$85,000.

Fig. 6.—Image of a “desmosome.” This section was “stained” with uranyl acetate. Mag. $\times$90,000.
Fig. 7.—Portion of adjacent cells of Yoshida ascites hepatoma and an intercellular canal. $er =$ endoplasmic reticulum; $g =$ Golgi complex; $i =$ granular inclusions; $m =$ mitochondria; $mc =$ cell membranes; $mv =$ microvilli projecting into canal CB; $CB =$ intercellular canal. Mag. $\times 24,000$. 
Fig. 8.—Portion of the cytoplasm of a poorly differentiated cell. $g =$ Golgi complex; $m =$ mitochondria; $n =$ nucleus; $P =$ Palade granules. Section of “Araldite”-embedded tissue, “stained” with uranyl acetate. Mag. $\times 24,900$. 
Fig. 9.—Portion of the cytoplasm of a relatively well differentiated cell. Smooth and rough vesicles of the endoplasmic reticulum are clearly visible. $em =$ cell membranes; $m =$ mitochondria. Mag. $X2,500.$
FIG. 10.—Portion of Yoshida ascites hepatoma cell. This image shows the very well developed Golgi complex. Small vesicles are seen to prevail with few large vesicles and no membrane system. l = lipide inclusions; n = nucleus. Mag. X18,700.

FIG. 11.—Area of Yoshida ascites hepatoma cell; image of undifferentiated hyaloplasm. The arrows show “filaments” between Palade granules. This section was “stained” with uranyl acetate. Mag. X90,000.

FIG. 12.—Area of a relatively well differentiated cell; image of “fenestrate lamellae.” Mag. X45,000.
Figs. 13, 14, 15.—Some aspects of mitochondria of Yoshida ascites hepatoma cells.

Fig. 13.—Typical mitochondrium. Mag. $\times 90,000$.
Fig. 14.—“Longitudinal” inner membranes. Mag. $\times 60,000$.
Fig. 15.—“Curved” inner membranes. Mag. $\times 75,000$.

Figs. 16, 17, 18.—Some aspects of swollen and vacuolated mitochondria. The picture seen in Figure 17 is common. Figure 18 shows the transformation of a mitochondrium into a vesicle. Sections of tissue embedded in “Araldite” and “stained” with uranyl acetate or, in Fig. 17, with potassium permanganate. Mag. $\times 60,000$. 

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Fig. 19.—Portion of Yoshida hepatoma cell. f = “fibrillar formation.” n = nucleus. Mag. ×15,000.

Fig. 20.—Micrograph at higher magnification showing the fine structure of filaments of the “fibrillar formations.” This section was “stained” with uranyl acetate. Mag. ×98,000.

Fig. 21.—A very dense body and a smaller ring-shaped one can be seen inside a vesicle. Section of “Araldite”-embedded tissue, “stained” with uranyl acetate. Mag. ×45,000.

Fig. 22.—Granular inclusion exhibiting a lamellar structure. Mag. ×65,000.
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