Some Ultrastructural Features of Yoshida Ascites Hepatoma

Joseph Locker, Peter J. Goldblatt, and Joseph Leighton

Laboratory of Electron Microscopy of the Department of Pathology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15263

SUMMARY

Several features of the ultrastructure of Yoshida ascites Hepatoma 7974, not previously reported, were observed in tumor cells withdrawn 7, 8, 9, and 17 days after intraperitoneal inoculation into JR rats. Tumor cells from the single animal which survived 17 days showed unique morphology, which perhaps indicated a very actively growing tumor. The cells examined after 7 to 9 days had a uniform morphology with dispersed ribosomes, annulate lamellae, and an unusual configuration of the endoplasmic reticulum. The 17-day-old tumor cells, the longest survival we observed in ascites growth, differed in that they had frequent, sometimes abnormal, mitoses, aggregated ribosomes, and at least one centriole showing an abnormal number of filaments in the radial subunits. Numerous virus-like particles were seen in this dimethylaminobenzene-induced hepatoma, both in the intercellular spaces and budding from the plasma membrane.

INTRODUCTION

As part of a continuing study on the implantation and growth of malignant cells injected intravenously into chick embryos (16), the fine structure of Yoshida ascites Hepatoma 7974 was studied immediately following withdrawal from the peritoneal cavity of the rat. This tumor line, originally derived from a hepatic malignancy induced in the rat by dimethylaminobenzene (DAB), grows as cell aggregates in the ascitic fluid (19). Although the ultrastructure of this line has been studied previously (12, 13, 23, 25) our examination, employing somewhat different technics of fixation and embedding, revealed several features not previously described which form the basis for this report.

MATERIALS AND METHODS

Tumor Inoculation. Adult white JR rats were inoculated intraperitoneally with 2.5 ml of ascitic fluid containing Yoshida hepatoma. An inoculum of this size killed 60% of the recipients within 8 days. By the 9th more than 90% of the inoculated rats were dead. An occasional animal survived longer; a single rat, representing the longest observed survival, lived 17 days. In all, ascitic fluid was studied from fourteen donors 7 days after inoculation, two each from 8 and 9 days and the single 17-day survivor. In each instance the hepatoma cells were sedimented in a clinical centrifuge at approximately 1,700 rpm for 10 minutes.

Electron Microscopy. The loose pellet of tumor cells was hardened in fixative for 15 minutes, then diced into approximately 1 mm cubes and fixed for an additional hour. Fixation was carried out in one of the following solutions at 0 to 4°C: s-collidine-buffered 2% osmium tetroxide (2); veronal acetate buffered 2% OsO₄ in 0.15 M sucrose (6); the formaldehyde-glutaraldehyde mixture of Karnovsky (14); and a fixative made up of three parts Karnovsky's mixture and one part 4% OsO₄ in a cacodylate buffer. After each fixation, the tissue was dehydrated through a graded series of alcohols and embedded in Epon-Araldite (J. W. Steiner, personal communication). Tissue was selected for examination in the electron microscope from one-μ-thick sections stained with toluidine blue. Gold to silver sections were cut on a Reichert OMY2 micromote with glass knives, picked up on 300 mesh copper grids, and stained with uranyl acetate (24) and lead citrate (21). All sections were examined with a Phillip's 100B electron microscope at an acceleration of 60 kv.

Additional blocks of tissue were fixed in Stieve's mixture and embedded in paraffin for light microscopic examination.

OBSERVATIONS

General Cytology. The tumor cells withdrawn 7, 8, or 9 days after inoculation showed the same morphology, but a number of differences were seen in the sample withdrawn after 17 days in the peritoneal cavity. While the tumor from the younger groups showed no mitotic activity, a large number of mitoses were seen in the 17-day-old tumor, amounting to approximately 10% of the population. A much larger number of degenerating cells was present in the 7- to 9-day-old tumor inocula than in the 17-day-old tumor.

In all groups the cells occurred primarily as aggregates of three to eight cells. Single cells represented fewer than 10% of the cells in the samples studied. The aggregates tended to be round or oval in outline, and individual cell borders were easily distinguished (Figs. 1–4). In the light microscope (Figs. 1, 2), the cells were seen to have a single large nucleus with one or more prominent nucleoli. The nuclei were usually cen-
trally located and round or oval, though irregular forms were seen. Usually there was at least one deep cytoplasmic invagination in each nucleus. In aldehyde-fixed material, a space was visible between individual cells in the aggregates (Fig. 2), while in material fixed primarily in osmium tetroxide, the cell borders were closely applied to one another (Fig. 1). A similar appearance was seen in low power electron micrographs (Figs. 3, 4). While the cell borders showed complex interdigitations, and microvillus formation in material fixed primarily in aldehydes (Figs. 4-6, 12), the addition of sucrose to a veronal buffer completely eliminated intercellular dilatation and showed instead a highly interdigitated cell border between tumor cells (Figs. 3, 7).

**Nuclei and Nucleoli.** As noted above, the nuclei tended to be round or oval and centrally placed, but often a deep cytoplasmic invagination was seen. The outer nuclear membrane was studded with ribosomes, and the inner and outer nuclear membrane formed frequent nuclear pores. In aldehyde-fixed material, the inner nuclear membrane appeared to be thickened (Figs. 5, 16), but this may represent aggregation of chromatin, rather than a true lamina fibrosa. In osmium-fixed material, the chromatin was more dispersed than in material fixed primarily in aldehyde, where the chromatin aggregated around the nucleolus and at the inner nuclear membrane. The interchromatinic substance was of low electron density and contained numerous aggregates of large interchromatinic granules. Occasionally perichromatin granules were also seen. The large nucleoli consisted of both particulate and fibrillar components disposed in a nucleolonna (Fig. 11). In addition, there were interspersed nucleolar vacuoles containing amorphous material of low electron density. In all instances a distinct association between the nucleolus and the nuclear membrane was seen (Figs. 4, 6, 7, 11). As illustrated in Figs. 6 and 11, the nucleolar mass appeared to be separated from the inner nuclear membrane by a thin layer of chromatin, but there was a distinct association with membrane invaginations.

**Plasmalemma.** The cell outline was roughly oval, and the plasma membrane was frequently thrown up into small microvillus projections. These projections were seen between adjacent cells making up a cell aggregate, as well as the periphery of cells found singly in the ascitic fluid (Fig. 19). As mentioned previously, primary osmium fixation, especially with added sucrose, reduced the number of villus projections between adjacent cells. The intercellular space was less evident. (Compare Figs. 3 and 4; also Figs. 5 and 7). However, small microvillus projections were evident even in material primarily fixed in Oso. Presumably the difference in morphology represents cell shrinkage in the hypertonic formaldehyde-glutaraldehyde-osmium fixative, though swelling in the osmium fixative is also a distinct possibility.

Junctional complexes were always observed adjacent to the borders of the islets, and desmosomes were frequently seen in the islet interior.

**Endoplasmic Reticulum and Golgi.** Ribosomal particles were attached to membranes or lay free in the cytoplasm. In tumor cells fixed after 7, 8, or 9 days of ascites growth, the ribosomes were attached to membranes or lay free in the cytoplasm. In tumor from the 17-day-old specimen, the ribosomes had a distinct aggregate pattern (polysomes) not seen in the younger tumor tissue. Cisternae of rough endoplasmic reticulum were apparent in all the cells of all age groups, but their structure varied greatly. A striking finding in numerous tumor cells from all groups was a tubular structure occupying the lumen of the endoplasmic reticulum (Figs. 12, 14-17). These tubules were occasionally multiple, but usually there was only one such structure running the length of the cisterna. This "tubular" endoplasmic reticulum was sometimes continuous with the normal rough endoplasmic reticulum and could also occasionally be seen to merge with annulate lamellae. Frequently these tubular structures were seen with one wall consisting of the nuclear membrane and often were observed near, but not attached to, a Golgi complex. Golgi complexes were small and numerous, and consisted of vacuoles, lamellae, and vesicles. Their appearance varied from almost circular to linear arrangements. Lipid vacuoles were frequently associated with Golgi regions. Centrioles were not seen in 7-, 8-, and 9-day-old cultures, but were occasionally seen in the older tumor tissue. As seen in Fig. 5, the centrioles consisted of radially arranged subunits, which in at least one cell were made up of more than the usual three microfilaments (see Fig. 5 insert).

**Single Membrane Limited Bodies.** In addition to lipid droplets, numerous small single membrane limited bodies were identified in the cytoplasm of tumor cells of all ages (Figs. 9, 10). Some of these, in presumably older tumor cells, were large and contained amorphous debris suggesting autophagy (Fig. 9) In other cells small single membrane limited bodies, some containing myelin-like figures, were seen (Fig. 10).

**Mitochondria.** All tumor cells contained numerous, small, irregularly shaped mitochondria. These had the usual inner membrane disposed in cristae, a dense matrix infrequently containing matrix granules, and a smooth outer membrane. Frequently, bulbous enlargement of the intercristal space was seen under all the modes of fixation employed (Fig. 16).

**Hyaloplasm.** The hyaloplasm was electron dense, although this was more noticeable after aldehyde primary fixation. In aldehyde-fixed material, numerous fine fibrils and microtubules were evident. In some cells perinuclear bundles of fibers were observed (Fig. 13). The majority of these filaments were approximately 60 to 80 A in diameter, with an apparent periodicity of 140-160 A (Fig. 13). Glycogen was not seen ultrastructurally, and could not be demonstrated in paraffin sections using the periodic acid-Schiff technic controlled with diastase digestion.

**Extracellular Space.** As illustrated in Fig. 13, numerous collagen fibrils, often in close association with the plasmalemma, were disposed around the tumor cells. Examination of the supernatant fluid after high speed centrifugation revealed a large amount of collagen in the ascitic fluid. In addition, numerous electron-dense bodies surrounded by a membrane consistent with virus particles were seen even in low power micrographs. These particles were found intercellularly (Figs. 4, 5, 8, 12, 15) and occasionally budding from cell walls. These virus-like particles had a total diameter of 1000-1400 A, with an inner dense core measuring approximately 800-900 A. They were not, however, clearly seen within the cytoplasm.

**Mitoses.** No mitoses were observed in tumor cells collected...
after 7, 8, or 9 days of ascitic growth. Several mitotic cells were seen, however, in the 17-day-old tumor (Figs. 14, 19, 20). The particular mitotic stages were difficult to differentiate. All stages, including metaphase chromosomes, showed patches of intact nuclear membrane. The cytoplasm of these mitotic cells showed granules resembling interchromatinic granules.

**DISCUSSION**

This investigation concentrated on the appearance of Yoshida ascites Hepatoma 7974 cells after 7, 8, or 9 days of intraperitoneal growth, since it is fluid from this stage which is injected intravenously into embryonated eggs to produce diffuse metastases (16). One unusual specimen consisted of fluid from a rat that survived to the 17th day. The reason for this long survival and for the high frequency of mitoses in the ascitic fluid of this animal is unknown. The mitotic index of this tumor resembles that of a very young inoculum (2—3 days) (19). The most likely explanation of this would seem to be the regrowth of the tumor following the death of the majority of the original cells.

Several ultrastructural investigations of the Yoshida ascites Hepatoma 7974 have been published describing general morphologic features similar to those presented here. Hoshino (12, 13) described this tumor as consisting of small compact islands of cells with irregular cell borders and microvilli projecting into the intercellular spaces. Terminal bars were prominent in the periphery of the islands. He also reported a progressive dilatation of the intercellular spaces toward the end of a transplant generation. Mitochondria and Golgi complexes were present but irregularly formed; the endoplasmic reticulum was sparse, as were free ribosomes. End-stage tumor frequently showed dark cells within the tumor islands. The nuclei, characterized by increased numbers of ribosomes, were large and irregular, and often contained several prominent nucleoli and deep nuclear invaginations. Yasuda et al. (25) described the tumor similarly. However, they found the reticulum of these cells to be almost exclusively of the agranular type and also reported the presence of numerous lipid vacuoles. Perinuclear bundles of cytoplasmic fibers have been described in a similar tumor, AH 130, both by these authors (25) and by Bairati (1). Finally, a paper by Usui and Kaziwara (23) described changes in the islet structure of AH 7974 following trypsinization. They reported that the terminal bars maintained contact even after trypsinization, although the intercellular spaces became greatly dilated. They also mentioned the presence of annulate lamellae in the tumor cytoplasm.

There is, of course, some question as to the origin of this hepatoma. It is not certain whether such DAB-induced hepatomas arise from the liver parenchyma or the bile duct epithelium. The absence of glycogen does not clarify this, because similar tumors, such as Yoshida AH 13, do have cytoplasmic glycogen (13). Furthermore, in studies to be published, we have found glycogen in hepatic metastases of Yoshida 7974 following the inoculation of rat ascites tumor intravenously in the chick embryo. Microvilli are reminiscent of both bile duct cells and liver parenchymal cells at the bile canalculus. The cellular origin of this tumor cannot be simply deduced from such morphologic features.

Virus-like particles have not previously been reported in a DAB-induced hepatoma. However, a close examination of the pictures published by Usui and Kaziwara (23) seem to show similar particles at low magnification in the intercellular spaces, though they did not comment on this. Although it is impossible to exclude virus as one of the factors inducing the malignancy, it seems more likely that these viruses are "passengers" growing within the tumor without necessarily having induced it. The frequent budding demonstrates clearly that the cells were infected. We have observed virus particles in each of 11 different generations of passage of the tumor examined with the electron microscope. These generations span a period of approximately one and a half years and represent over 70 generations of tumor transplantation.

We have observed several unusual configurations of rough endoplasmic reticulum including a spiral-like configuration and rough endoplasmic reticulum with an apparent tubule in the lumen. Szollosi (22) described similar endoplasmic reticulum in several different kinds of mammalian oocytes. They have also been described in a human myxosarcoma (15). Spiral configurations were observed in rat and mouse oocytes, and tubular structures were seen in oocytes from the guinea pig and the Mongolian gerbil (22). In oocytes such structures were also observed adjacent to the nuclear wall and appear to be morphologically identical to the configurations seen in these tumor cells. The general appearance is that of a cysterna of rough endoplasmic reticulum which, when cut longitudinally, appears to contain a tubule running down the entire length of the vesicle, sometimes for several microns. This tubule is about 300 Å in diameter, and the interior appears to show a periodicity, though this is difficult to resolve. This tubular structure may be multiple, but usually only one is seen. In some cases it looks as if two separate cysternae of endoplasmic reticulum, studded with ribosomes on both surfaces, appear to merge to form this structure, but in other cases no associated endoplasmic reticulum can be seen. This may indicate, as previously suggested (15, 22), that this is not really a tubule, but rather consists of the associated membrane profiles of parallel cysternae, which have been degranulated. Whether this is the case, it is convenient to continue referring to such structures as "tubular endoplasmic reticulum". The cysternae that show multiple tubules closely resemble the Golgi complex, except for the fact that the outer membrane is studded with ribosomes. In addition, they have been seen to merge with small portions of annulate lamellae in several micrographs. In either case the significance is not apparent. These structures may in fact represent a synthesis of some cytoplasmic membranous component by the rough endoplasmic reticulum. Finally, similar configurations have also been seen in dividing Walker tumor cells (5) and HeLa cells (9).

Annulate lamellae have been observed in a wide variety of cells, including hepatomas induced by a number of carcinogens (17, 18). They are seen in embryonic cells but have not been reported in embryonic liver. The significance of this structure in relation to the malignancy of the tumor cells is not apparent, but annulate lamellae are frequently observed in hepatomas (17).
Joseph Locker, Peter J. Goldblatt, and Joseph Leighton

Cytoplasmic fibers have been observed in a number of different tumors, including several Yoshida ascites hepatomas (1, 13, 25). These fibers seem to show periodicity that is significantly different from the periodicity of collagen fibers seen in the same ascites fluid and differs also from such fibers as amyloid (100 Å) (7) or fibrin (240 Å) (11). There does seem to be a relationship between the number of cytoplasmatic fibers and the age of the cell. In older tumors occasional dark cells can be observed, and most of the darkness of the cytoplasm can be attributed to the extremely large number of such fibers in the cytoplasm. It is conceivable that such an accumulation of fibers represents some sort of degenerative process. These cells, however, are not periodic acid-Schiff positive in paraffin section, and this probably does not correspond to the periodic acid-Schiff positive 200—400 Å fibrillar material seen by Hoshino in AH 604.

The single centriole that was clearly observed showed an abnormal number of microtubules in its radial subunits. The significance of this finding is unknown, but it must be remembered that highly malignant tumors, including this one, very frequently show abnormal mitosis.

As pointed out previously (4), morphologic differences between normal and neoplastic cells are subtle at best and are usually quantitative rather than qualitative. Cancer cells resemble more embryonal forms in general. The presence of an abnormal centriole may be related to the bizarre mitotic figures seen. The finding of virus-like particles, though of unknown significance, raises questions of cocarcinogenic effects in this chemically induced malignancy.

REFERENCES

Figs. 1-4. A comparison of aldehyde-osmium and osmium-isotonic sucrose fixation showing with light and electron microscopy how observation of intercellular relationships in this system is dependent on the method of fixation.

Fig. 1. 7-day-old tumor. Veronal acetate-osmium-sucrose fixation. The arrows indicate some of the close cell membrane associations that can be seen following the use of this fixation. Toluidine blue, × 650.

Fig. 2. 7-day-old tumor. Formaldehyde-glutaraldehyde-osmium fixation. Arrows indicate dilatation of the intercellular spaces following aldehyde fixation. Toluidine blue, × 650.

Fig. 3. Same specimen as in Fig. 1. The intercellular border is clearly tight and interdigitating. The mitochondria (M) are irregular, as are the lipid vacuoles (L). Two junctional complexes (T) can be seen. Even at this low magnification, intercellular particles (V) are evident. × 4800.

Fig. 4. Same specimen as in Fig. 2. Following aldehyde fixation, the intercellular spaces became dilated. A spiraling profile of rough endoplasmic reticulum is apparent, as are several nuclear invaginations (N). Chromatin aggregates (Chr) can be seen about the nuclei. Also visible are a desmosome (D), a junctional complex (T), a lipid vacuole (L), and intercellular particles (V). Note the diffuse, nonaggregated ribosomal background. × 7200.

Fig. 5. Portion of the cytoplasm of a 17-day-old cell. Note the distinct ribosomal aggregates. An underlying cell apparently projects upward into the plane of section. Intercellular virus particles are numerous (V). There are also several intracytoplasmic particles which may be viruses (stars). The arrows indicate “tubular” rough endoplasmic reticulum approaching a small profile of annulate lamellae (AL). The background contains a network of fine fibrils (F). Other profiles of rough endoplasmic reticulum (ER) and a centriole (CE) are also visible. Formaldehyde-glutaraldehyde-osmium fixation. × 22,500. Insert, enlargement of the centriole in Fig. 5. Arrows show radial subunits with irregular numbers of microtubules. Stars show possible satellite structures. × 60,000.

Fig. 6. 17-day-old cell showing extensive nuclear invagination and a large number of interchromatinic granules (ICG). Note also the perinuclear bundle of fibers (F) and balloonized cristae in some of the mitochondria. Formaldehyde-glutaraldehyde-osmium fixation. × 11,200.

Fig. 7. Highly interdigitated intercellular border between two 17-day-old tumor cells. Intercellular virus (V) and irregular lipid vacuoles (L) are also evident. Veronal acetate-osmium-sucrose fixation. × 9,800.

Fig. 8. Intercellular virus particles. Arrow indicates one such particle budding from the plasma membrane. The star shows a dense aggregate at the cell membrane which may be an earlier stage of virus formation. Formaldehyde-glutaraldehyde-osmium fixation. × 76,000.

Fig. 9. Large autophagic vacuole in a degenerating 7-day-old tumor cell. Osmium-s-collidine fixation. × 6,800.

Fig. 10. Several varieties of cytosome in another degenerating 7-day-old tumor cell (C1, C2, C3, C4). Two spiraling configurations of rough endoplasmic reticulum (arrows) are also visible. Osmium-s-collidine fixation. × 11,800.

Fig. 11. Detail of the nucleolus from a 7-day-old tumor cell. Nuclear invaginations (N), chromatin (Chr), perichromatin granules (PG), nucleolar vacuoles (NV), and particulate (P) and fibrillar (F) components of the nucleolus are discernible. Formaldehyde-glutaraldehyde-osmium fixation. × 15,600.

Fig. 12. Cytoplasm of a 17-day-old cell showing large numbers of fibrillar bundles (stars) throughout the cytoplasm. Arrows indicate “tubular” rough endoplasmic reticulum. A lipid vacuole (L), a desmosome (D), a junctional complex (T), and intercellular virus (V) are also visible. Formaldehyde-glutaraldehyde-osmium fixation. × 11,800.

Fig. 13. A perinuclear fibrillar bundle at high magnification in a 7-day-old cell. Arrow indicates an area where the periodicity of these fibers can be clearly seen. Formaldehyde-glutaraldehyde-osmium fixation. × 48,000.

Fig. 14. A long clear profile of “tubular” rough endoplasmic reticulum in a late telophase cell on Day 8. Cytoplasmic granules (CG) resembling interchromatinic granules may be seen. Note the mitotic chromatin (Chr) with a specialized area (star) at the nuclear border. Note also the fine cytoplasmic fibrils at the cell border. (F). Veronal acetate-osmium-sucrose fixation. × 12,400.

Fig. 15. Arrow indicates a segment of “tubular” rough endoplasmic reticulum. Two Golgi complexes (G) and a short profile of annulate lamellae (AL) are also apparent. Note the thicker inner nuclear membrane. Formaldehyde-glutaraldehyde-osmium fixation. 7-day-old tumor. × 29,200.

Fig. 16. Two profiles (arrows) of “tubular” rough endoplasmic reticulum with the nuclear membrane as the inner limiting surface. Note the proximity of nuclear pores. A Golgi (G) and a double profile of annulate lamellae (AL) are also visible. 7-day-old tumor. Formaldehyde-glutaraldehyde-osmium fixation. × 20,100.

Fig. 17. An atypical segment of rough endoplasmic reticulum showing a double “tubule.” Arrows denote the ribosome studded outer membranes. 7-day-old tumor. Formaldehyde-glutaraldehyde-osmium fixation. × 21,500.

Fig. 18. A typical Golgi complex (G). Compare with Fig. 17. 7-day-old tumor. Formaldehyde-glutaraldehyde-osmium fixation. × 25,200.

Fig. 19. A 17-day-old cell in mitosis, apparently atypical early telophase. Arrows indicate areas of membrane formation about the chromatin mass. Stars show concentrations of microtubules which may be spindle fibers. Note the masses of interchromatin-like granules in the cytoplasm (CG). Formaldehyde-glutaraldehyde-osmium fixation. × 8,000.

Fig. 20. Irregular metaphase. In this 17-day-old cell, the chromosomes are clearly spread out over most of the cytoplasm. The figure shows a small portion of the cell containing several such chromosomes. Arrows indicate areas of nuclear membrane formation associated with large ribosomal aggregates, an event which would not be seen in a normal cell at this stage of mitosis. Note the cytoplasmic granules (CG) resembling interchromatinic granules. Formaldehyde-glutaraldehyde-osmium fixation. × 8,200.
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