SUMMARY

An intranuclear canalicular system connecting the nuclear envelope to the nucleolus has been found in interphase cells of the Novikoff hepatoma in both the ascitic and the solid forms. Throughout the nucleoplasm, flattened canaliculi are seen in sections as parallel membranes covered by thin layers of chromatin material. Membrane-bounded tubules exist in direct contact with the nucleolus, where they are often stacked in parallel array and embedded in an amorphous matrix substance. Those intranuclear membranes disappear at metaphase and reappear at telophase. It seems likely that the canaliculi are formed by deep invagination of only the inner layer of the nuclear envelope and reach into the nucleolus. The interspace of intranuclear canaliculi is continuous with the perinuclear cisterna of the nuclear envelope, which, in turn, is confluent with the cisternae of the rough endoplasmic reticulum.

INTRODUCTION

The specific development of membranous structures in the nucleolus has been demonstrated only in human endometrium during the secretory phase of the menstrual cycle (1, 4, 14, 19) and in salamander (12) and crayfish (9) oocytes during early stages of oocyte growth. In a wide variety of transplantable tumor cells (3, 5, 10, 11, 24), intranuclear lamellae have been noted to exist in the nucleoplasm, and there is evidence that they are connected with the inner membrane of the nuclear envelope. Little attention, however, has been paid to the association of membranes with the nucleoli of neoplastic cells. Although the fine structure of the nuclei of Novikoff hepatoma cells has been extensively examined by Unuma and Busch (21) and Smetana et al. (17), no intranuclear membranes were reported by these investigators. In a strain of this tumor line, membranous components have been found in the nucleolus as well as in the nucleoplasm (2, 8).

The primary purpose of this paper is to describe in detail the fine structure and distribution of intranuclear membranes in cells of the Novikoff hepatoma during the logarithmic phase of tumor growth.

MATERIALS AND METHODS

Cells of the Novikoff hepatoma were maintained in an ascitic form by weekly i.p. injections into male Sprague-Dawley rats. Ascites cells were harvested between 6 and 8 days after implantation, suspended in cold Dulbecco's phosphate-buffered saline, and then pelleted free from blood cells by low-speed centrifugation. Subcutaneous transplants were also used for solid tumors in the same strain of rats. Ten days after implantation, samples were taken from several portions of only those solid tumors with no recognizable necrosis.

For a fine structural examination, the cell pellets or tissue pieces were placed in cold 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 min and postfixed in cold 1% OsO₄ in the same buffer for 1 hr. They were dehydrated in ethanol and embedded in Epon. Thin sections were cut on a Porter-Blum MT-l microtome, stained with both uranyl acetate and lead citrate, and examined under a JEM-7A electron microscope.

RESULTS

The interphase nuclei of the hepatoma cells are quite similar in appearance, regardless of whether the tumor was obtained in the ascitic or the solid form. The nuclei vary in size from 8 to 25 μ with irregular invaginations and projections. The nuclear envelope is well defined and consists of outer and inner membranes. The nuclei contain prominent nucleoli, which are often multiple in number and irregular in shape. At a low magnification, the chromatin material appears to be rather unevenly and sparsely distributed throughout the nucleoplasm. Chromatin clumps are often disposed along the nuclear envelope and surrounding the nucleoli.

Fig. 1 shows an interphase nucleus containing a number of canalicular membranes in the nucleolus as well as in the nucleoplasm. These structures will be referred to as intra-
nuclear canaliculi. Their profiles, lined by a single membrane, are readily distinguishable from those of intranuclear cytoplasmic inclusions, which are lined by a double-layered nuclear envelope. Individual canaliculi are seen dispersed throughout the nucleoplasm, coursing in a straight, curved, or spiral manner. Their elongated profiles sometimes extend more than 5 μ. They are also found near, touching, or in direct continuity with the nuclear envelope. In close association with the nucleolus, there is a striking accumulation of membranous components (Fig. 2). As seen in Fig. 3, several canaliculi extend from the nuclear envelope towards the nucleolus. Fig. 4 illustrates that both the nuclear envelope and the nucleolus are actually connected by a canaliculus which traverses the nucleoplasm. In some cases, those intranuclear canaliculi aggregate to form multiple layers, while in others they branch or anastomose (Fig. 1).

Regardless of the plane of the section, the canalicular system present in the nucleoplasm is most frequently seen as 2 parallel membranes which are separated from one another by a space of 20 to 100 μ, with an average of 50 μ (Fig. 3). This profile suggests that the membrane-limited structure is in the form of lamellae, rather than of tubules. Each membrane has a smooth surface free of ribosome particles and, like the inner layer of the nuclear envelope, it is accompanied by a continuous layer of chromatin material. Two parallel membranes are not interrupted by nuclear pores or annuli.

The most conspicuous development of intranuclear membranes is seen adjacent to or directly connected with the nucleolus (Figs. 2 and 5 to 7). As reported previously by Unuma and Busch (21) and Smetana et al. (17) for this tumor cell, the nucleolus reveals 2 main types of zones, granular and fibrous. Each zone consists predominantly of ribosome-like granules about 15 nm in diameter and fine fibrils 2 to 8 nm in diameter, respectively. Towards the interior of the nucleolus, canaliculi are often distended and assembled in groups of 2 to 8 units (Figs. 2 and 7). Some canaliculi converge at more or less regular intervals (Fig. 5). Frequent findings of circular profiles indicate that most of the membranes are in the form of tubules rather than of lamellae. When the tubules are cut in cross-section, a hexagonal honeycomb pattern is evident (Fig. 6). The longitudinal sectioning produces an array of parallel paired membranes (Fig. 7). The diameter of individual tubules ranges from 50 to 300 nm. The interspace of canaliculi contains only a variable amount of amorphous substance (Fig. 2). Intranuclear membranes are not associated with condensed chromatin material. Instead, there is an occasional accumulation of an amorphous matrix substance of moderate density (Fig. 6). The membranes are also seen in direct contact with nucleolar granular and fibrous components (Fig. 7).

At the periphery of the nucleus, canalicular membranes merge with the inner membrane of the nuclear envelope. The direct connection of both membranes is clearly seen in Fig. 8. When the intranuclear membrane is in direct contact with the inner nuclear membrane, the interspace is continuous with the perinuclear cisterna. The latter is often seen in continuity with the cisterna of the rough endoplasmic reticulum (Fig. 9).

From the count of interphase nuclei in sections of 20 samples obtained from either ascitic or solid tumors, the frequency of those showing intranuclear canaliculi varies between 25 and 55%. In a given nucleus, there is usually more than 1 intranuclear canaliculus, with all canaliculi widely separated from one another. Membranous structures occur with a high frequency in nuclei larger than 20 μ in diameter, in which up to 60 profiles have been counted in a single nuclear section. The cells possessing a significant canalicular system do not show any degenerative changes in either nuclear or cytoplasmic components.

In dividing cells, chromosomes are easily recognized, standing out clearly against a background of granular material. During prophase, intranuclear membranes are still present but tend to segregate from the chromosomal material (Fig. 10). When the nuclear envelope disappears at metaphase, the intranuclear membranes are not seen in the chromosomal masses. At telophase, when the nuclear envelope begins to reappear at the surface of chromosomes, canalicular membranes are also seen penetrating deeply into chromosomal masses (Fig. 11). These membranes may persist within the nucleoplasm after complete reconstruction of the nuclear envelope.

**DISCUSSION**

The ultrastructural analysis of nuclei of the transplantable hepatoma cells has revealed the presence of a new membranous component. Examination of sections taken from different planes indicates that the intranuclear component occurs in the form of flattened canaliculi dispersed throughout the nucleoplasm. The membranes are found to be connected with the inner nuclear membrane, on one hand, and the nucleolus, on the other. In the nucleolus, they are organized in the form of tubules. In the most favorable micrographs, one observes a continuous canalicular system connecting the nucleolus with the perinuclear cisterna of the nuclear envelope. The latter is often continuous with the cisternae of the rough endoplasmic reticulum (23). Such a confluent system between the nucleus and the cytoplasm may exist, at least temporarily, in all interphase cells of the Novikoff hepatoma during the logarithmic phase of tumor growth.

The development of intranuclear membranes is a unique phenomenon in the transplantable hepatoma, since they have not been reported in either normal or pathological hepatocytes (13, 16) or in the cells of primary hepatic tumors (18). In describing the fine structure of the hepatoma cells, previous investigators (6, 7, 15, 17, 22) did not report the presence of intranuclear membranes in the earlier transplants. It seems improbable that such a prominent structure had escaped observation in the past because of their liability to disruption by preparative procedures. However, a close examination of electron micrographs of the nuclei of Novikoff hepatoma cells, published by Unuma and Busch (21) and Unuma et al. (20), seems to show a similar profile of intranuclear membranes, particularly adjacent to the nuc-
leoli, although they did not comment on this structure. In the same strain used in the present investigation, Babai et al. (2) recently reported the presence of a tubular structure in the nucleolus as well as in the nucleoplasm. In our strain of the transplantable tumor, it is conceivable that the complete intranuclear canalicular system has appeared in relatively recent transplants of hepatoma cells. This would be regarded as an example of the ultrastructural differentiation in neoplastic cells after a long period of serial transplantation.

This study contributes information to the question of the origin of the intranuclear membrane. Its smooth surface, lamellar appearance, and peripheral accumulation of heterochromatin are various aspects that are identical to those of the inner layer of the nuclear envelope (23). Again, similar to the nuclear envelope, it lacks continuity during the cell cycle. Both structures disappear at metaphase and reappear at telophase. These features and the finding of a direct continuity between both membranes suggest that the canalicular system is derived from the inner nuclear membrane. This suggestion is made on the assumption that the inner nuclear membrane is extensively infolded and such folds reach into the internal region of the nucleolus.

Electron micrographs suggesting deep invagination of the inner nuclear membrane into the nucleoplasm were first demonstrated by Hoshino (5) in a Yoshida ascites hepatoma. Occurrence of intranuclear lamellae was also reported in an Ehrlich ascites tumor (24), a Yoshida ascites hepatoma (11), a Rous sarcoma (3), and a 6C3HED ascites lymphoma (10). It thus seems clear that intranuclear membranes are of widespread appearance among rapidly growing and malignant tumors. Although the distribution and the configuration of the lamellae are very different among these tumors, they are commonly related to the inner nuclear membrane. It is probably valid to consider that, in Novikoff hepatoma cells, as well as other neoplastic cells, the specific control mechanism normally regulating the elaboration of the inner nuclear membrane is defective and that such a defect causes the unusual development of intranuclear membranes. Observations indicating a continuity of the interspaces of intranuclear canaliculi with the perinuclear cisterna of the nuclear envelope may provide a morphological basis for interrelation between the nucleus and the cytoplasm: throughout this system, substances, capable of moving across the membrane could be exchanged in either direction to support the activity in the nucleus or to transfer the nuclear products into the cytoplasm. It has been shown clearly that the canaliculi are particularly organized as a complex of membranes with nucleolar components. On the basis of evidence that the nucleolus of this cell type is actively engaged in the formation of cytoplasmic ribosomes (17, 20–22), one would expect that the canalicular system could facilitate the function of the nucleolus.

The possibility of the transport of the nucleolar products via a canalicular system has been discussed by a number of investigators who have observed membranous components associated with the nucleolus (1, 2, 4, 9, 12, 14, 19). Studying the nucleolar "channel" system of human endometrium, Terzakis (19) has found granular components inside of the membrane-bounded tubules and suggested that the channel represents a passage for the ribosome subunits from the nucleolus into the cytoplasm. In Novikoff hepatoma cells, the interspace of canaliculi does not contain any major components of the nucleolus, but only an amorphous substance. Furthermore, a radioautographic examination of such cells labeled with uridine-5-3H for different periods of time does not provide any evidence for preferential localization of labeled RNA in the canalicular system (8). These facts suggest that in Novikoff hepatoma cells transfer of newly synthesized RNA through the canalicular system does not take place or is negligible. Other possible roles of the canalicular system, including the transport of RNA precursor from the perinuclear space into the nucleolus, are currently under investigation in this laboratory.

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REFERENCES

Electron micrographs of thin sections of Novikoff hepatoma cells in ascites or solid form. Specimens were fixed in glutaraldehyde and OsO₄, embedded in Epon, and stained with uranyl acetate and lead citrate. Marker lines, 1 μ in Figs. 1 to 5, 10, and 11, and 0.1 μ in Figs. 6 to 9.

Fig. 1. A portion of the nucleus (N) and cytoplasm (C) of an ascites cell. In the nucleolus (NO), stacks of elongated canaliculi are seen (arrows). Numerous lamellae (arrow) are found either near the middle of the nucleus, or along its border. Cytoplasmic inclusions (c) appear to be formed by the invagination of the double membranes of the nuclear envelope. X 9500.

Fig. 2. Enlargement of a part of Fig. 1. In close association with the nucleolus, paired membranes are assembled in roughly parallel array. Some membranes are in direct contact with fibrillar and granular components of the nucleolus. The interspaces of canaliculi contain a moderately dense matrix substance. X 33,000.

Fig. 3. A portion of an ascites cell nucleus and cytoplasm. Intranuclear canaliculi extending from the nuclear envelope to the nucleolus. The paired membranes of canaliculi are accompanied by a continuous layer of chromatin just as is the inner membrane of the nuclear envelope. X 18,000.

Fig. 4. A portion of an ascites cell nucleus. A continuous canaliculus connecting the nucleolus with the nuclear envelope. Arrow, place at which the inner nuclear membrane is continuous with that forming the canaliculus. X 41,000.

Fig. 5. A portion of an ascites cell nucleus and cytoplasm. Intranucleolar tubules are sectioned either longitudinally or transversely; the latter sectioning produces circular patterns. The membrane complex is embedded in a nucleolar amorphous component and is further surrounded by nucleolar granular components. A canaliculus is continuous with the invaginated nuclear envelope (arrow). Small cytoplasmic invagination (C). X 28,000.

Fig. 6. A portion of an ascites cell nucleus. Intranucleolar tubules are sectioned transversely. They are seen embedded in an amorphous matrix substance (A) of a moderate density. The area is surrounded by nucleolar granular components. Line, 0.1 μ X 120,000.

Fig. 7. A portion of an ascites cell nucleus. Elongated profiles within the nucleolus may correspond to a longitudinal section of tubules. Each membrane shows a unit membrane structure. The nucleolus shows fibrous (F) and granular (G) regions. Line, 0.1 μ X 70,000.

Fig. 8. A portion of a cell from the solid hepatoma. The inner layer of the nuclear envelope is deeply infolded into the nucleoplasm, while the outer layer remains on the surface of the nucleus. Thus, the interspace of an intranuclear canaliculus is continuous with the perinuclear cisterna of the nuclear envelope. Line, 0.1 μ X 70,000.

Fig. 9. A portion of a cell from the solid hepatoma. Arrow, place at which the outer membrane of the nuclear envelope is continuous with the membrane of rough endoplasmic reticulum. Line, 0.1 μ X 80,000.

Fig. 10. A portion of a late prophase nucleus from the solid hepatoma. Granular components of the nucleolus (NO) are seen in association with chromatin material. At the initial stage of nuclear envelope breakdown, the intranuclear membranes seem to be segregated from the chromosomal masses (arrows). X 50,000.

Fig. 11. Telophase of an ascites cell. The nuclear envelope encloses the coalescent mass of chromosomal material. The beginning formation of an intranuclear membrane can be seen at the arrow. X 30,000.
An Electron Microscope Study of Intranuclear Canaliculi in Novikoff Hepatoma Cells

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