An Electron Microscope Study of Intranuclear Inclusions in Mouse Liver and Hepatoma*

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ABSTRACT

An electron microscope study of intranuclear inclusions which occur in giant cells in a transplantable mouse hepatoma and in enlarged liver cells in mice fed a diet containing bentonite demonstrates that these inclusions are formed by invaginations of the nuclear envelope, and corroborates a previous histochemical study which revealed that the contents of the inclusions are of cytoplasmic origin.

In the hepatoma cells the intranuclear inclusions are abundant, small, and situated close to the border of the nucleus, and there are wide openings from the cytoplasm into the invaginations whose contents include lipid droplets, ergastoplasm, and structurally normal mitochondria.

In the enlarged liver cells the inclusions are fewer in number, generally much larger than those in the hepatoma, hence they extend deeper into the nucleus, and the interior is continuous with the cytoplasm through only a small opening. Some normal ergastoplasm is present within the inclusions but all other constituents are abnormal. Both normal and degenerating mitochondria occur in the cytoplasm but only degenerating ones are found within the inclusions.

Both types of inclusions arise in greatly enlarged cells in which an attempt is made to maintain the normal nuclear surface/nuclear volume ratio by the development of the invaginations of the nuclear envelope.

INTRODUCTION

In an earlier publication (8) we described the histochemistry of intranuclear inclusions which occur consistently in giant cells in a transplantable mouse hepatoma and in greatly enlarged liver cells in mice maintained on a diet containing bentonite. The inclusion bodies were shown to contain materials of cytoplasmic origin which were segregated within the nucleus. It was postulated at that time that the inclusions could be formed either by an invagination of the nuclear envelope and engulfment of portions of the adjacent cytoplasm as described by Kleinfeld et al. (6) and Wessel (11), or by the penetration of small or plastic cytoplasmic elements through the nuclear envelope via its pores, there to accumulate between the distended chromosomes of the interphase nucleus. Inclusions of the second type would not be bounded by the double membrane of the nuclear envelope. Intranuclear inclusions without limiting membranes have been described by Himes and Pollister (5) and Binggeli (2). An electron microscope study of the intranuclear inclusions in our material has revealed that they are certainly formed by an invagination of the nuclear envelope and that the inclusions are certainly of cytoplasmic origin because they contain cytoplasmic elements such as mitochondria and ergastoplasm.

Material and Methods

The hepatoma in which intranuclear inclusions occur, hepatoma SS3, arose spontaneously in a C3H/StWi mouse and is maintained by serial subcutaneous passages (8, 7) in mice of the same strain. The mice are maintained on Purina laboratory chow in temperature-controlled rooms. Most of the hepatoma cells are small and contain no intranuclear inclusions, but a few cells...
randomly distributed in the tumor contain very large nuclei which, in turn, contain numerous small intranuclear inclusions. Giant liver cells were induced by maintaining young mice of the BUB strain for 60 to 80 days on a methionine-rich basal diet mixed with an equal amount of bentonite (8, 7, 12). All hepatic parenchymal cells throughout the lobule become greatly enlarged. The correspondingly enlarged nuclei usually contain one or more inclusions which may vary greatly in size but which frequently are larger than normalized whole nuclei.

Small blocks of tissue, 1 mm.³ or less, were cut in a few drops of fixative and then fixed at 5°C. for 1 and 2 hours in 1 and 2 per cent aqueous solutions of osmium tetroxide plus 0.4 M sucrose buffered at pH 7.4 to 7.8 with veronal acetate. The tissue was dehydrated in increasing concentrations of ethyl alcohol and embedded in an 85:15 mixture of n-butyl methacrylate and methyl methacrylate plus 1 per cent luproca at 55°C. Thin sections were cut with the Porter-Blum microtome, mounted on collodium and carbon-coated grids, and examined with an RCA EMU3D electron microscope.

OBSERVATIONS

Hepatoma SS3.—Light microscope studies (8) showed that the intranuclear inclusions were closely associated with the nuclear envelope and bound by Feulgen-positive and basophilic rims, staining reactions which are characteristic of the nuclear envelope. Sudanophilic lipid droplets were always present within the inclusions and in a few there also was histochemically demonstrable acid and alkaline phosphatase and esterase activity. The inclusions were only faintly basophilic, if at all, and no glycogen was detected within them.

Electron micrographs have revealed that the inclusions are bounded by a distinct double membrane that is continuous with the nuclear envelope (Figs. 2 and 3). At the point of invagination of the nuclear membrane, the opening is relatively wide so that there is good continuity between the cytoplasm and the contents of the invagination or "inclusion." All of the inclusions examined contain lipid droplets (Fig. 1). Occasional intranuclear lipid droplets occur around which membranes cannot be distinguished (Fig. 3), but these also appear to be formed by nuclear envelope invagination (Fig. 4). Some inclusions contain mitochondria which appear structurally normal (Fig. 3). Occasionally, there are irregular, membranous structures which correspond to the endoplasmic reticulum of the cytoplasm; the granular component of the ergastoplasm is very sparse in the inclusions. Cytoplasmic constituents which have not been found in the inclusions are glycogen and elements of the Golgi apparatus, but it is recognized that traces of these constituents in inclusions could easily escape recognition. The Golgi apparatus is well developed in the adjacent cytoplasm and appears to be associated with numerous vesicles. Cytoplasmic glycogen is aggregated into large discrete masses. Because phosphatase and esterase activities were found in some of the larger inclusions, we looked for but did not find structures corresponding to the descriptions of lysosomes (10). Nucleoli are very large and complex in these cells, but they appear to have no particular relationship with the inclusions (Fig. 4).

Enlarged Hepatic Cells.—The intranuclear inclusions in the enlarged liver parenchymal cells also are bounded by a Feulgen-positive, basophilic rim. The contents include ribonuclease-digestible basophilic substances, occasional sparse accumulations of glycogen and of hemosiderin, and intense histochemically demonstrable acid and alkaline phosphatase, esterase, and β-glucuronidase activities (8). Sudanophilic lipids and succinic dehydrogenase activity were not detected in the light microscope study.

Electron micrographs show that these inclusions are bounded by a double membrane (Fig. 7). Examination of numerous sections through each of many inclusions frequently revealed connections with the nuclear envelope (Figs. 5 and 6), hence it seems that these inclusions also develop by an invagination of the nuclear envelope. Unlike those in the hepatoma, however, the opening from the cytoplasm into the invagination or inclusion is relatively small in relation to the size of the inclusion (Fig. 6). The complex, large nucleoli in these cells are not associated with the inclusions in any way.

The structures within the inclusions are highly variable and they sometimes resemble but are never identical with those in the cytoplasm. Normal ergastoplasm and mitochondria are always found in the cytoplasm but as the cells increase in size various changes take place. The cytoplasm sometimes becomes filled with small spherical vesicles which appear to be pinched off from the ergastoplasmic sacs, although the vesicles have few if any RNA-protein particles on their surfaces. The intranuclear inclusions always contain a few well defined ergastoplasmic sacs or cisternae (Figs. 5 and 6) and they often contain small spherical vesicles like those in the cytoplasm.
and closely associated with it there are frequently granules which are identical with those described in abnormal livers by deHarven and Friend (4). Such granule-containing vesicles occur within some of the inclusions. The most striking abnormal elements of the cytoplasm are dense bodies which are irregularly oval in outline, variable in size but usually as large or larger than normal mitochondria, split in several places by spindle-shaped spaces of very low density, and often enclosed in a thin membrane (Fig. 8). These bodies resemble the damaged mitochondria isolated from rat liver homogenates by Novikoff (9) (Figs. 2 and 7) and we, therefore, interpret them to be degenerating mitochondria. These degenerating mitochondria appear first in the general vicinity of the bile canaliculi and eventually are dispersed throughout the cytoplasm. They are also found within some of the large intranuclear inclusions (Fig. 5). On the other hand, equally large and abundant normal mitochondria are in the adjacent cytoplasm (Fig. 8) but they have not been seen within the inclusions. One constituent of some inclusions that is rarely found in the cytoplasm is a complex, concentric system of membranes of unknown origin. Occasional accumulations of ferritin particles occur both in the cytoplasm and in the inclusions. These were probably responsible for the positive hemosiderin reaction at these sites which we reported previously (8). Glycogen has not been identified within the large inclusions and lipid droplets are only rarely found there, but some lipid droplets occur independently within the nucleoplasm and limiting membranes around them cannot be discerned (Figs. 5 and 6). Finally, a few small dense bodies have been seen in both the cytoplasm and inclusions which appear to be the size of lysosomes but we have not been able to detect any internal structure that would identify them with certainty (10).

**DISCUSSION**

In both the enlarged liver cells and hepatoma cells all of the intranuclear inclusions except those completely filled by lipid were surrounded by a clearly discernible double membrane. When numerous sections through a single inclusion were examined, it was usually found that at one point this double membrane was continuous with the double membrane or envelope of the nucleus and that an opening was present connecting the interior of the inclusion with the cytoplasm. Although double membranes could not be resolved around the inclusions that were entirely occupied by a lipid droplet, one such inclusion well within the nucleus was found to which the double nuclear membrane extended, then disappeared as if obscured by or dissolved in the lipid. Therefore, it seems that all of our inclusions, like those of Kleinfeld et al. (6) and Wessel (11), unquestionably represent invaginations of the nuclear envelope.

In the large hepatoma cells, the invaginations or so called “inclusions” have relatively wide-mouthed openings in relation to their small size. Portions of the endoplasmic reticulum have been seen extending through some of these openings. Furthermore, large elements of the cytoplasm, the mitochondria, are engulfed by the invaginations and retain their normal structure. Therefore, either a normal exchange of metabolites through these relatively wide openings must be possible, or the invaginations are only transient structures, constantly forming and disappearing. This can be determined only by a study of living, cultured cells. In either case this seems to be an excellent mechanism for increasing the ratio between the surface of the nucleus and its volume (8). Wessel (11) demonstrated the presence of normal mitochondria, ergastoplasm, and Golgi apparatus in intranuclear inclusions in the Crocker sarcoma, and Bernhard (1) reported that “deep invaginations” of the nuclear envelope, which appear to contain normal cytoplasmic constituents, occur frequently in cancer cells.

In the enlarged liver cells the majority of the inclusions differ from those in the hepatoma cells by their much greater size; they may indeed occupy most of the nuclear space. In contrast to this large size, each inclusion or invagination appears to be connected with the cytoplasm through only a relatively narrow, duct-like opening. Furthermore, although occasional segments of apparently normal ergastoplasm usually are seen in these inclusions, most of the contents are abnormal. No normal mitochondria are present, but degenerating ones are fairly common. The invaginating nuclear envelope must entrap only moribund mitochondria (which is unlikely since normal and degenerating ones are randomly mixed in the cytoplasm), or engulfed mitochondria of normal structure must degenerate promptly in
an unfavorable environment. The presence of whorls of membranes also suggests a transformation of cellular constituents in an unfavorable environment (3). Similar narrow openings and abnormal contents were described in livers of thioacetamide-treated rats (6) and colchicine-treated mice (11). If these inclusions formed in order to compensate for an altered nuclear surface-volume ratio as we have previously proposed (8), their postulated role in increasing the effective area of the nuclear surface seems to have been lost. This may be one of the reasons that all of these giant cells became moribund and eventually died (12). In this connection Wessel (11) makes an interesting point that the livers in which intranuclear inclusions occur are abnormal, but the tumor cells in which they occur, e.g. in his work, the Crocker ascites sarcoma, are healthy, growing cells. The cytoplasmic constituents of his liver cell inclusions were abnormal but those of his tumor cells were normal. Comparison of liver inclusions and those in a hepatoma in our material corroborates his findings completely.

A few small invaginations, close to the nuclear envelope, were also found in the enlarged liver cells and these had wide-mouthed openings like those in the hepatoma through which cytoplasmic elements might readily enter or leave. Indeed, normal ergastoplasm and a degenerating mitochondrion were observed within one of them. It is possible, therefore, that all newly developed inclusions have this form, and that the size of the opening diminishes as the inclusion enlarges.

REFERENCES
7. Leduc, E. H., Metastasis of transplantable hepatomas from the spleen to the liver in mice, Cancer Research, in press.

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FIG. 1. Low magnification of a giant cell in hepatoma SS3 to demonstrate the numerous, small, intranuclear inclusions arrayed contiguous to the nuclear envelope. Two of the inclusions in this figure are shown at higher magnification in Fig. 3. X 9,000.
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FIG. 2. An intranuclear inclusion in hepatoma SS3 formed by the invagination of the double membrane of the nuclear envelope (arrow). Nucleoplasm is on the left, cytoplasm with mitochondria, on the right. The inclusion contains a lipid droplet and membranes of the endoplasmic reticulum which protrude into the wide-mouthed opening leading into the cytoplasm. × 26,000.

FIG. 3. Two intranuclear inclusions in the same section as Figs. 1 and 2 which contain structurally normal mitochondria and lipid droplets and which are bounded by a double membrane presumably continuous at some point with the nuclear envelope (arrow). Lipid droplet (L) in upper left corner had no visible limiting membrane. Nucleoplasm is on the left of the nuclear envelope, cytoplasm on the right. × 26,000.
Fig. 4. Two lipid droplets (L) within the nucleoplasm in a hepatoma SS3 cell have no readily discernable limiting membranes, but an invagination of the nuclear envelope (arrow) leading to one of them suggests that a double membrane may be or once was present but it has become obscured in some way by the lipid. On the far right, a large nucleolus. X 18,000.

Fig. 5. Portion of a nucleus (nuc) of an enlarged liver cell which contained several intranuclear inclusions. The large inclusion at the bottom of the page contains ergastoplasm (thin arrows), degenerating mitochondria (wide arrows), and other abnormal constituents. Its limiting membrane was continuous with the nuclear envelope in another section of the same nucleus. Above it is the edge of a second inclusion whose limiting membrane in another section became continuous with the invagination of the nuclear envelope seen at I. Lipid droplets (L) were numerous in this nucleus. X 15,000.
(Leduc and Wilson: Intranuclear inclusions)
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Fig. 6. An intranuclear inclusion (incl) (in an enlarged liver nucleus (nuc)) whose contents are continuous with the cytoplasm (cylo) through a narrow opening formed by the invaginated nuclear envelope. The inclusion contains ergastoplasm and vesicles and granules presumably derived from or associated with the ergastoplasm. A portion of a second inclusion (incl) is at the right. X 20,000.

Fig. 7. An enlargement of the boxed portion of Fig. 6 to show the double membrane of the nuclear envelope (arrows) which invaginates and encloses the cytoplasmic elements within the intranuclear inclusion. X 47,000.
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Fig. 8. Low magnification of an enlarged liver cell to demonstrate a large inclusion (incl) within the nucleus (nuc) whose contents are largely abnormal. In the cytoplasm (cyto) are normal mitochondria (M) and degenerating ones (arrow). × 16,000.
(Leduc and Wilson: Intranuclear inclusions)