Electron Microscopic, Histologic, and Histochemical Features of the Walker Carcinoma*

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SUMMARY

The morphology, including ultrastructure and histochemical features (particularly those related to enzymatic activity), of subcutaneous and artificially induced hepatic metastases of the Walker tumor has been investigated. The results of examinations by light and electron microscopy indicate that the designation of this tumor as a carcinoma appears more accurate than as a sarcoma or carcinosarcoma.

Both local and hepatic growths qualitatively disclose a similar enzymatic pattern histochemically. Noteworthy is the failure to demonstrate succinic dehydrogenase and little cytochrome oxidase activity, although DPN- and TPN-linked diaphorases are well demonstrated within the tumor cells. The relationship is not uncommon to a variety of malignant neoplasms and indicates the altered metabolism of such cells. Diaphorase as well as acid phosphatase, nonspecific esterase, lipase, and β-glucuronidase activities which were also noted were less intense within tumor cells of hepatic metastases than in the subcutaneous growths.

The electron microscopic appearance of subcutaneous growths and hepatic metastases of the Walker carcinoma were similar, resembling the appearance of a variety of other malignant tumors. Attention is called to the presence of pseudopodal cytoplasmic extensions between tumor and hepatic cells in the hepatic lesions. On the other hand, cell contact among tumor cells appeared as a more simple effacement of such cells.

Similar enzymatic and ultrastructural features were noted in rapidly growing hepatic metastases induced by partial hepatectomy.

The Walker tumor of the rat is widely utilized in experimental cancer research. Its ease of transmission, lack of regression or strain specificity, rarity of spontaneous metastases, and successful explantation to tissue culture and the chorioallantoic membrane of the chick have allowed for diversified investigations concerning neoplastic growth. However, it is surprising to learn that aside from the thorough morphological studies of Earle in 1935 (2) no detailed analysis of the histological, ultrastructural, or histochemical features of this neoplasm have been recorded. Further, one gains the impression from the varied terminology employed in its designation—viz., tumor, carcinoma, and, more commonly, carcinoma—of its precise identification has not been accomplished. It should be noted in this regard, however, that the tumor as first observed by Walker in 1928 in a pregnant female rat about 10 months of age exhibited a definite adenocarcinomatous structure (2). Its origin from breast was highly suspect. During its subpassage this architectural pattern disappeared, although the cellular elements, for the most part, resembled the parent tumor. Earle (2) considered the neoplasm as a carcinoma from the results of his histological and tissue culture studies. Although cells in long-term tissue cultures were purely epithelial, their reinoculation into rats resulted in tumor growth of a fibrosarcomatous pattern. However, he interpreted this change to be the result of an elongation of the epithelial components and not to represent true fibrosarcoma. Shrek and Avery (18) considered the Walker tumor to repre-
sent a sarcoma and noted its carcinomatous transformation when cultivated on chick embryo chorioallantoic membrane. Their designation of these tumor types was based principally on the spindle-shaped appearance of the neoplastic cells in subcutaneous growths in the rat and polygonal configuration in growths on the chorioallantoic membrane.

During the past 2 years we have been utilizing the Walker tumor for studies concerned with factors influencing the development of artificially induced hepatic metastases. These studies have necessitated over several hundred subpassages of this tumor. It has been our impression, gained from the examination of numerous cell suspensions and selected samples of artificially induced hepatic growths as well as local growths, that fundamentally the Walker tumor is comprised of a round to polygonal cell with variable numbers of spindle forms. The purpose of this report is to present the results of various tinctorial and histochemical procedures and electron microscopic study of the Walker tumor as observed in subcutaneous growth and hepatic metastases.

MATERIALS AND METHODS

Five Walker tumors were excised 2 weeks following the subcutaneous implantation of 250,000 tumor cells in the groins of five adult female Sprague-Dawley rats weighing 200 gm. This tumor has been propagated in our laboratory by this procedure for the past 2 years. Macroscopically, the tumors measured approximately 2.0 cm. in diameter, and their cut surfaces were for the most part moist and homogeneously tan, with small foci of hemorrhage. Hepatic metastases were obtained from another five rats 2 weeks following the direct intraportal injection of 250,000 Walker tumor cells. The method of preparation of tumor cells for injection has been described previously (6). In addition, metastases were obtained from three animals 2 weeks after partial hepatectomy. The latter has been noted previously by us to increase the incidence as well as size of such artificially induced metastatic deposits (5).

Portions of tumor from both primary and metastatic growths were fixed in Zenker acetic fluid (ZA), buffered formalin (F), as well as quickly frozen on dry ice (FF). Tissues prepared by the latter method were stored at -80°C and sectioned in a cryostat at this temperature. Electron microscopy was performed with a Philips EM 100 microscope on sections of tumors prepared from small, 1-mm. cubes of tumor fixed in osmic acid and imbedded in a mixture of methyl and butyl methacrylate.

The following histologic and histochemical procedures (fixation designated in parentheses) were performed according to the methods described in Lillie's Histologic Technic and Practical Histochemistry (10), unless otherwise indicated.

**OVERSIGHT METHODS**

1. Hematoxylin and eosin (ZA)
2. Masson trichrome (ZA)
3. Wilder's reticulum method (ZA)
4. Phosphotungstic acid hematoxylin (PTAH) (ZA)

**MUCOPOLYSACCHARIDES**

1. Periodic acid-Schiff with and without antecedent treatment with barley malt diastase (pH 6.0 in phosphate buffer; 1 hr. at 37°C) (F)
2. Alcian blue (F, FF)
3. Thionin, 1:10,000, pH 4, with and without antecedent treatment with ribonuclease (F, FF)
4. Rinehart-Abul Haj method (F, FF)

**ENZYMES**

1. Succinic dehydrogenase according to Rosa and Velardo (15) (FF)
2. Cytochrome (G-nadi) oxidase (FF)
3. Alkaline phosphatase according to the methods of Gomori and Pearse (14) (FF)
4. Acid phosphatase according to methods of Gomori and Pearse (14) (FF)
5. Beta-glucuronidase
6. Nonspecific esterase
7. DPN and TPN diaphorases with substrates containing DPHN and TPNH (14) (FF)
8. Lipase according to method of Gomori

**LIPIDES**

1. Oil red O and sudan black B for neutral lipides (F, FF)
2. Fischler method for fatty acids (FF)
3. Schultz modification of Lieberman-Burchard reaction for cholesterol and/or esters (FF)

**RESULTS**

The morphologic appearance of the Walker tumor in routinely prepared sections examined by light microscopy was similar to that described in detail previously (2). The examples examined in this study were comprised principally of round and polygonal tumor cells with large, round, vesicular nuclei with prominent nuclear membranes and nucleoli (Fig. 1). Mitoses including abnormal types were not infrequent. Cell cytoplasms were hematoxylinophilic and cell borders for the most part distinct. The tumor cells were arranged in a pavementsed manner which was most evident in suitably fixed tissue. Occasional spindle cells
The ultrastructure of the Walker tumor in subcutaneous and hepatic sites was similar. The cells varied in configuration from round to polygonal, with spindle and transitional forms occasionally evident (Figs. 4, 5). Nuclei were large and frequently exhibited an irregular contour with invaginations. The nuclear membrane in favorable sections appeared to be comprised of two distinct borders. Nuclear chromatin was finely dispersed, and nucleoli were not unusual in their appearance, containing aggregates of electron-dense granules as well as vacuoles. The former appeared to be similar to the granules of nuclear chromatin. Occasional slits were evident within the nuclearplasm and often could be traced to the nuclear membrane. On rare occasion similar-appearing tubular structures in the cytoplasm could be seen indenting the nuclear membrane (Fig. 6). The cytoplasm of the tumor cells contained relatively few organelles. Mitochondria were round to oval, with occasional angulated forms being noted (Fig. 7). Cristae were not abundant, and some empty mitochondria were evident. The endoplasmic reticulum was for the most part comprised of scanty, isolated tubules. Only rarely was this structure well developed (Fig. 8). In both instances they contained condensations of Palade granules which were also moderately dispersed throughout the tumor cell cytoplasm. The cytoplasm of tumor cells also contained small vesicles measuring up to 500 Å in diameter, which only on rare occasion were condensed within the cytoplasm along with small tubules comprising a well defined Golgi zone (Fig. 5). Rare cells contained solitary large lipide droplets within their substance. In none of the many micrographs examined were replicate particles similar to those characteristic of virus encountered.

Apposition of tumor cells appeared for the most part as a simple contact of cytoplasmic borders, but occasionally focal areas separated by lacunae were noted. On the other hand, in the metastases apposition of tumor and liver cells appeared more complex, although in many areas simple contact also prevailed. Not infrequently pseudopodal evaginations of tumor cell cytoplasm extended into the cytoplasm of hepatic parenchymal cells after the fashion of the parts of a jigsaw puzzle (Figs. 7, 8). Absolute fusion of the cytoplasms of these cells by this apparent bridging, however, was not conclusively discerned. Lacunar interruption of effacement of tumor and hepatic cells, although similar in appearance to that noted between tumor cells, was more frequent and larger in the former situation. Occasionally portions of the cell membrane forming the boundary of such spaces had a beaded appearance. Except for apparent compression, hepatic cells adjacent to tumor cells did not disclose alteration. No ultrastructural differences were noted in those hepatic lesions of larger size and apparently more rapid growth than was observed in tumors of control animals.

DISCUSSION

The morphologic features of the Walker tumor examined in this study warrant its designation
as a carcinoma. The effacement of cell borders, which is readily appreciated in routinely stained sections of suitably fixed tissue as well as in electron micrographs, is in keeping with fundamental concepts concerning the arrangement of epithelia. Further support of its epithelial nature is provided by the lack of demonstrable protoplasmic fibrillary extensions in sections stained by the phosphotungstic acid hematoxylin and Masson techniques. Such structures may be readily identified in some mesenchymal cells, notably fibroblasts and smooth muscle cells. The argentophilic reticulum pattern exhibited by the Walker tumor is also compatible with its epithelial derivation. Although isolated as well as larger areas of these neoplasms reveal spindle cells, suggesting a sarcomatous component, it appears noteworthy that these cells and foci are tinctorially, morphologically, and ultrastructurally similar to that observed in more clearly carcinomatous areas as recounted above. The presence of transitional forms between the spindle and round or polygonal tumors cells further indicates that the former represent morphologic variants of the more common prototype. Edwards and associates (3) observed spindle cells in their electron microscopic study of a human carcinoma of lung transplanted to hamster cheek and considered them to represent immature forms of the more common cellular type. These considerations indicate that the designation carcinosarcoma so commonly employed for this tumor is invalid.

The enzymatic pattern encountered in the subcutaneous implants of the Walker carcinoma, as well as the artificially induced hepatic metastases, reflects the altered metabolic pathways exhibited by this neoplastic tissue. The lack of a histochemically demonstrable succinic dehydrogenase system and glucose-6-phosphatase indicate an aberration in the citric acid cycle and possibly glucose-phosphate metabolism in these neoplastic cells. The association of depleted succinic dehydrogenase but active diaphorase activity noted in the Walker tumor has been observed previously in a variety of epithelial and some mesenchymal neoplasms explored by these histochemical technics by Monis and associates (13) and in colon carcinomas by Wattenberg (19, 20), may represent a common enzymatic pattern of malignancy. It has been considered as a mechanism which could facilitate rapid glycolysis, a well recognized phenomenon of malignant neoplastic cells, without leading to lactate formation (20). The depletion of cytochrome oxidase and absence or depletion of succinic dehydrogenase have also been observed in a variety of tumors by quantitative technics (16) and have been considered as indicative of the malignant nature of these neoplasms. Wattenberg (20) observed weak histochemical reactions for oxidase but intense succinic dehydrogenase activity in the base of the crypts of benign lesions of the colon. On the other hand, in malignant tumors, particularly infiltrating carcinoma, low succinic dehydrogenase activity was evident. The significance of the positive reactions for acid phosphatase and nonspecific esterase and lipase in this or other tumors remains to be elucidated. It is of interest, however, that the former has been observed in breast tumors (10) and that the Walker carcinoma historically was considered to be derived from such tissue. Although admittedly it is difficult as well as hazardous to attempt to quantitate the results of histochemical reactions, nevertheless, it was apparent that the metastatic lesions observed within the liver which were procured simultaneously with the local growths disclosed less intense reactions than were observed in the cells from the latter, although qualitatively the enzymatic patterns were similar. No ultrastructural alterations were apparent which might account for this discrepancy of reactivity. It is possible that the decrease in reactivity observed within the hepatic metastases reflects a more malignant nature of these cells than those of the primary growth. It appears worthy of note that no difference in the intensity or distribution of enzymatic activity was evident in rapidly growing hepatic metastases from animals with partial hepatectomy and those from intact animals. This experience is similar to that of biochemical analyses of this relationship in neoplasms in general (8).
Fig. 4.—General electron microscopic appearance of Walker carcinoma. Simple effacement of cytoplasmic membranes is evident. Tumor cells contain irregular nuclei and relatively few cytoplasmic organelles. X5500.

Fig. 5.—Electron micrograph of spindle cell of Walker carcinoma at left. The cytoplasmic particles are similar to those observed in round and polygonal cell types as noted in Fig. 7. A lacuna is noted at site of cell contact. X12,500.

Fig. 6.—Nucleus of tumor cell revealing indentation of nuclear membrane by cytoplasmic tubule. Other tubules are observed within the nucleus. The nucleolus is not unusual in appearance. X9500.
Fig. 7.—Electron micrograph of portion of cytoplasm of Walker carcinoma cell disclosing type of cell contact with adjacent tumor cell (arrow) and cytoplasmic organelles. The presence of a well formed Golgi apparatus in this cell is an unusual occurrence in the Walker carcinoma. X33,000.
FIG. 8.—A well developed endoplasmic reticulum in the cytoplasm of a Walker carcinoma cell. This is an unusual feature of such cells. More often the endoplasmic reticulum appears as sparse interrupted tubules as noted in Fig. 7. A double nuclear membrane is apparent (arrow). \( \times 38,500 \).

FIG. 9.—A large pseudopod (surrounded by arrows) extending from the tumor cell cytoplasm into that of an adjacent liver cell is apparent. \( \times 20,000 \).

FIG. 10.—Appearance of cell contact between a tumor and liver cell revealing frequent pseudopodal connections. \( \times 9000 \). The fine structure of some of these configurations is depicted in the insert. \( \times 21,500 \).
Elevated β-glucuronidase activity noted in the Walker tumor has been observed to be increased in a variety of cancers (7).

The electron microscopic appearance of the Walker carcinoma is not unlike that observed in a variety of human and experimental malignant neoplasms (1, 3). The disparity of organelles including well formed endoplasmic reticulum and vesicular aggregates characteristic of the Golgi apparatus was striking. Mitochondria were occasionally angulated and contained few cristae but did not show other significant abnormalities. This appears significant, since succinic dehydrogenase was not evident within the tumor cells, although many exhibited diaphorase and mild oxidase activities. Since all these enzymes are intramitochondrial doubt is raised concerning the validity of using these enzymatic reactions as an assay of mitochondrial integrity as has been suggested (14). The absence of distinguishing ultrastructural changes in the more rapidly growing hepatic metastases is similar to the observations made concerning the enzymatic activity of these tumors.

The electron microscopic appearance of the sites of cell contact between tumor and hepatic cells is of interest. Although in many instances simple contact of cell membranes was observed as was evident between tumor cells, the relationship between the latter and hepatic cells was distinctly more complicated. The presence of pseudopodial cytoplasmic extensions of the tumor cells into adjacent hepatic cells is reminiscent of a similar configuration considered to be indicative of ameboid motility and invasive nature of some tumors (4, 12, 16). It has also been suggested that it represents a mechanism for increasing the surface area of these cells so as to allow for an increase in nutrition from the surrounding environment (12) or is related to the phenomenon of pinocytosis (10). If the situation observed in the hepatic metastases of the Walker carcinoma is analogous to the above observations confined principally to the local growths of tumors, the possibility that many of the tumor cells derive nutrient from adjacent hepatic cells is worthy of consideration and further exploration.

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