A Transplantable Bile-Secreting Hepatocellular Carcinoma in the Rat

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SUMMARY
A bile-secreting, transplantable hepatocellular carcinoma, with bile plugs in the canaliculi, is described in the rat. All transplants of this tumor, which has been carried for 6 generations, have shown bile pigment. This finding has not been previously reported. The tumor also contained iron and ceroid pigments. Bile pigment and iron were also present in the kidneys, in addition to a third unidentified pigment, which was probably lipofuscin.—J. Nat. Cancer Inst. 26: 891-897, 1961.

BILE SECRETION into the canaliculi between adjacent liver cells has frequently been noted in primary human hepatocellular carcinoma, but curiously, it is more often found in the metastases. It has also been noted in well-differentiated liver cells in a metastatic teratocarcinoma of the testis in a supraclavicular lymph node (1). The morphology of induced hepatic tumors in rats has been described in detail by various workers (1-10); however, the finding of bile pigment in these tumors has not to our knowledge been reported. Although this finding has been attributed to Kinosita (2), bile pigment is not described in his paper which has been quoted by others (1, 4). Edwards and White (4) observed material suggesting intercellular biliary casts which were not pigmented in unstained sections and were often intimately associated with collagen. They concluded that these were strands of connective tissue that for some reason did not take the specific stains.

In the present work on liver carcinogenesis with the use of N-2-fluorenyldiacetamide, a semisynthetic diet, and AXC strain rats, several hundred primary hepatocellular carcinomas have been studied. Approximately 50 of these were transplanted isologously, homologously, or heterologously, each for 3 or more generations (11).

Many bile casts within canaliculi were observed in 1 line and smaller amounts of bile pigment in 2 other lines of transplanted hepatocellular carcinoma. The former is described in detail here.

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3 This research was started at the University of Kansas and completed at the University of Maryland, the present address of the author.
HISTORY OF TUMOR

This bile-secreting tumor arose in a male A × C rat, which received 0.025 percent N-2-fluorenyldiacetamide in the semisynthetic diet of Morris, No. 272 (12). The diet consisted of 300 g commercial casein, 2275 g skim-milk powder, 6152 g ground, hard spring wheat, 200 g Brewer's yeast, 200 g desiccated whole liver powder (Wilson), 140 g sodium chloride, 10 g ascorbic acid, 13 g ferric citrate, 100 g cod-liver oil, and 610 g corn oil. The carcinogenic diet was administered for 4 weekly periods, each separated by 1 week of the same diet without carcinogen. Thereafter the rat was continued on the basal diet until autopsy was performed 10 months later. At the time of autopsy the rat weighed 269 g and the liver weighed 32.4 g. There was a 3.4 × 2.6 × 3.1 cm gray, nodular, firm tumor in the left lobe of the liver. Microscopic study disclosed a well-differentiated hepatocellular carcinoma with invasion of the portal vein and many metastases to the lungs. There was minimal cirrhosis in the liver and a few small cysts were seen. Multiple sections failed to reveal bile pigment within the tumor or in the pulmonary metastases.

The hepatoma that was transplanted subcutaneously to 3 male A × C rats grew in 2 of them. During the second generation it was transplanted into 3 female A × C rats and again grew in 2 rats. Bile plugs were observed in both the first and second transplanted generations. The tumor from one of the second-generation animals was labeled H-35 and was used in this study.

METHODS AND PROCEDURES

The third generation of the tumor was transplanted subcutaneously into the right groin of 25 A × C female rats 3 to 4 months of age, weighing 150 to 175 g. The rats were fed laboratory pellets. The animals were weighed and the tumors measured at 2- to 3-week intervals throughout the experiment. When the rats were killed at 4 months the serum and urine appeared icteric. The Glenner stain for bilirubin (13), and the Oil Red O stain for fat were performed on unfixed tumor and kidney. Tumor tissue was fixed in cold acetone, and alkaline-phosphatase studies were carried out according to the sodium glycerophosphate-calcium chloride technique of Gomori (14). The heart, lungs, kidneys, spleen, thymus, salivary gland, ovaries, adrenals, urinary bladder, and axillary, retroperitoneal, and groin lymph nodes were fixed in 10 percent formalin and stained with hematoxylin and eosin. The following stains were carried out on the tumor and kidney: periodic acid-Schiff (PAS), Verhoeff acid fast, Oil Red O, Sudan Black-B, Gomori iron, Stein bilirubin, Hall bilirubin (15), Glenner bilirubin, Masson's trichrome, Gridley's reticulum, and Kutlick's ferric-iron method for bilirubin.
RESULTS

At 1 month the transplants measured 0.2 to 0.5 cm in greatest diameter; at 2 months, 1.3 to 3.0 cm; at 3 months, 4.5 to 5.0 cm; and at 4 months, 5.0 to 6.5 cm. In 3 rats the transplant did not grow. The average weight of the animals at time of death was 106 g. The tumors varied in weight between 20 and 80 g but averaged 38 g.

Gross Findings

The tumors were usually cystic and this was evident by external palpation. The cystic areas measured up to 2 and 3 cm and contained reddish-brown or dark-purple fluid which was bile-stained. The tumor tissue resembled liver tissue partitioned by wide bands of darkly bile-stained connective tissue. Areas of the tumor 3 to 4 mm in diameter were also bile-stained, usually adjacent to the fibrous tissue (fig. 1). The corticomedullary junction of the kidney was brownish-green and the cortex was brown. After formalin fixation, the bile-stained areas of tumor and the corticomedullary junction of the kidney were bright green; the remainder of the cortex of the kidney was light green.

Microscopic Findings

The tumor had a trabecular pattern with double cords of cells separated by prominent vascular spaces which were often lined by Kupffer cells. In areas it grew in solid sheets. There were many well-developed canaliculic structures between adjacent tumor cells. Often these contained olive-green casts which in frozen sections were dark green (fig. 4). The cells of the tumor resembled hepatic parenchymal cells with abundant eosinophilic cytoplasm, central vesicular nuclei, prominent single nucleoli, and marginal chromatin lining the nuclear membrane (fig. 5). Intra-cytoplasmic inclusions were not observed. Reticulum was confined to perivascular areas and was negligible among tumor cells. The cystic areas were lined by compressed and atrophic tumor cells. Islands of tumor were separated by broad bands of collagen containing reticulum and scattered round macrophages with small nuclei. Tumor cells adjacent to these areas sometimes contained granules or small globules which were colored by Oil Red O in frozen sections but not in paraffin sections. PAS reaction revealed positive granules in the cytoplasm which were removed by previous diastase digestion.

Alkaline-phosphatase reaction was present in the sinusoidal linings and nuclei of Kupffer cells by the Gomori technique. There was also very finely granular reactive material in the pericanalicular areas which served to identify those structures in many areas throughout the tumor. An incidental finding consisted of somewhat coarser granules in the cytoplasm adjacent to the sinusoids in occasional cells. They were not further identified.
Tumor cells appeared to be secreting small granules of olive-green pigment into the canaliculi (fig. 3). The olive-green casts in the canaliculi were always adjacent to the bands of connective tissue in the tumor. Nearby macrophages in the connective tissue also contained similar pigment. The pigment, which was light green or greenish-yellow on unfixed tissue, stained bright emerald green in unfixed tissue with the Glenner stain and in fixed tissue with the Hall stain. In unstained paraffin sections it was olive green. In paraffin sections with Kutlick's and Glenner's stains it deepened but remained olive green. It was negative for iron and isotropic between crossed polaroids.

Many macrophages contained coarse clumps of brown pigment which gave a positive reaction for iron. In others there was canary-yellow pigment, which was strongly acid-fast, PAS-positive, and fluorescent in ultraviolet light, and stained with Oil Red O both in frozen and paraffin sections.

Within the fibrous stroma large venules were surrounded irregularly by several layers of large tumor cells containing iron. Bile pigment was present within small canaliculi. Often these cells were separated from the lumen of the vessel by a single layer of endothelial cells. A few recent thrombi were noted within the vessels.

In the outer zone of the cortex of the kidney there were homogeneous spherical bodies in the cytoplasm and lumens of proximal tubules. These bodies varied from eosinophilic to olive green and measured 7 to 15 μ in diameter (fig. 2). They were bright yellow in unfixed frozen sections and light brown to olive in formalin-fixed frozen sections stained with Oil Red O. They appeared greenish yellow in unstained paraffin sections and pale green to olive with bilirubin stains.

There was also in the kidney brown pigment which often appeared clumped within the cytoplasm of many proximal tubular cells. In the inner zone of the cortex multiple brown droplets were observed in almost every cell lining the proximal and distal tubules. They remained brown with all stains. They occasionally stained faintly in fat stains of paraffin sections. Iron stains were negative.

In the cytoplasm of the proximal tubules in the outer zone of the cortex there were scattered, fine, iron-containing particles, which stained very light brown on hematoxylin and eosin. Brown clumps within stromal macrophages were also positive for iron.

There was extramedullary hematopoiesis and small amounts of hemosiderin in the pulp of the spleen. The regional lymph nodes contained hemosiderin and ceroid pigment within macrophages. In the lung of one animal there was a tiny metastasis. The remaining organs were not unusual.

**DISCUSSION**

Pigment studies have been carried out on the third-generation transplants of a well-differentiated hepatocellular carcinoma which acquired
the ability to secrete bile pigment in the first-generation transplant to an isologous host. In addition to olive-green bile casts, there were two additional pigments in the tumor. Both were predominantly within macrophages in the wide bands of connective tissue. One was in the form of granular, brown clumps which contained iron, apparently hemosiderin. The other pigment was in the form of somewhat clumped, canary-yellow, amorphous globules, and represented ceroid, an unsaturated lipide which had undergone auto-oxidation and polymerization. Often hemosiderin and ceroid were present within the same macrophages. There were also lipides within macrophages which did not remain after paraffin embedding.

The kidney contained three types of pigment. There were globules within the lining cells and lumens of the proximal convoluted tubules, particularly in the outer cortex. They were proportional to the number of bile plugs present within the tumor and are interpreted as bile pigment or bile-stained hyaline droplets. In the lining cells of the proximal convoluted tubules there were small scattered particles which gave a positive reaction for iron. There was also iron-containing material within the stromal macrophages. The third pigment, golden brown, granular material, was unidentified. It was observed within proximal convoluted tubular cells and stromal macrophages of the cortex. It seems likely that it was lipofuscin pigment.

Bile pigment has not been observed in primary or transplanted liver tumors in animals. Edwards and White (4) described both iron and ceroid pigment, but did not note bilirubin in their experiments with tumors in rats. The iron within macrophages in the tumor was explained on the basis of hemorrhage or hemolysis within the tumor. The ceroid that was at the periphery of the primary tumors was believed to be a remnant of the cirrhotic liver. Ceroid is present in experimental dietary cirrhosis in rats (16-18), in mice treated with carbon tetrachloride (19), in vitamin-E-deficient animals (20), and in fish affected with a wide variety of pathologic diseases (21); it can be produced in vitro by the mixture of unsaturated fats and red blood cells (22) or proteins (23).

Kinosita (2) observed iron within kidney tubular cells in rats with liver tumors. Fairhall and Miller (24) described pigment in the kidneys of rats receiving compounds of lead and concluded that it was de-ironized blood pigment. Similar pigment was reported by Edwards and White (4) in animals with hepatic tumors. It is also seen in kidneys of animals with other transplantable tumors.

Attempted correlation of the findings suggests that the bile pigment secreted by the tumor was carried in the blood stream and then excreted by either the liver or the kidneys, or both. It was present as globules within the proximal convoluted tubules of the kidney. Iron pigment from hemorrhage or hemolysis entered the circulation and, when the level was great enough in the blood, it could be identified within the kidney as such. Ceroid pigment probably did not enter the circulation but was carried in macrophages to regional lymph nodes.
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PLATE
FIGURE 1.—Tumor transplant resembling liver tissue partitioned by wide bands of darkly green-stained connective tissue. Bile-stained areas of tumor tissue adjacent to connective tissue and cysts containing bile-stained fluid.

FIGURE 2.—Globules of bile pigment or bile-stained hyaline droplets within cells, and lumen of proximal convoluted tubule of cortex of kidney with periodic acid-Schiff. $\times 700$. These vary from brown to olive green and sometimes orange, with hematoxylin and eosin stains.

FIGURE 3.—Tumor cells with bile pigment within the cytoplasm, often surrounded by a clear halo, and a bile cast in the lumen. Hematoxylin and eosin. $\times 810$

FIGURE 4.—Canalicular structure between adjacent tumor cells containing a bile cast. Hematoxylin and eosin. $\times 700$

FIGURE 5.—Tumor cells resembling hepatic parenchymal cells; adjacent band of connective tissue containing many macrophages (not shown) with ceroid and iron pigment and few with bile pigment; bile casts within canaliculi. Hematoxylin and eosin. $\times 415$