ON MESENCHYMAL REACTION OF RAT LIVER IN AZO-DYE CARCINOGENESIS, ESPECIALLY ON THE FORMATION OF CARTILAGE. SPECIAL CONSIDERATION ON THE MIXED TUMOR OF THE LIVER.
(With Plates XV—XVII)

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In the vast series of azo-dye rat hepatoma experiments since Yoshida and Sakaki (1935), Kinoshita (1937) first stressed importance of initial mesenchymal proliferation in the periportal area in the course of formation of cholangiomas by DAB, Maruya (1941) saw occasional myxomatous structures, Edwards and White (1941) myxomatous structure with scattered islands of cartilage, and Mulay & Firminger (1952) bone and cartilage formation. Richardson et al. (1951) found among 57 tumor bearing rats fed 3'-methy-4-dimethyl-aminazo-benzene sarcoma 5 times and bone and cartilage formation 4 times. No detailed description or discussion has been attempted concerning mesenchymal reaction seen in the liver of azo-dye fed rats.

Material for this study consists of 8 rat liver tumors, in three series of experiments with DAB-feeding as listed below. One additional tumor is a second generation transplant of a DAB-hepatoma. Details of the experimental series are reported elsewhere.

<table>
<thead>
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<th>Table 1. List of Materials</th>
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<tr>
<th>Rat (diet)</th>
<th>Lobe</th>
<th>Size</th>
<th>Carcinoma</th>
<th>Mesenchyma</th>
<th>Days of Exp.</th>
<th>DAB mg.</th>
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<td>R-39</td>
<td>2nd</td>
<td>1.0  x 1.0</td>
<td>Trabecular hepatoma with glands (osteoid)</td>
<td>+</td>
<td>182</td>
<td>566</td>
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<tr>
<td>(Rice)</td>
<td></td>
<td>1.0  x 1.0</td>
<td>Mucin (+)</td>
<td></td>
<td></td>
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<tr>
<td>R-35</td>
<td>5th</td>
<td>5.0  x 5.0</td>
<td>Cholangiocarcinoma</td>
<td>-</td>
<td>173</td>
<td>673</td>
</tr>
<tr>
<td>(Rice)</td>
<td></td>
<td>5.0  x 5.0</td>
<td>Muc. (+)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>R-28</td>
<td>2nd</td>
<td>4.5  x 4.0 x 2.5</td>
<td>Cholangiocarcinoma + Ad. hep. glandular Muc. (+)</td>
<td>+</td>
<td>156</td>
<td>541</td>
</tr>
<tr>
<td>(Rice)</td>
<td></td>
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FINDINGS

(A) R-39-Z: An 1 cm sized tumor in the middle of the second lobe of the liver was proved to be a composite mass, mainly of trabecular hepatoma with development of tubular structures, and a broad sheet of highly cellular mesenchymal tissue, well intermingled with the former and playing the part of the stroma for the carcinomatous epithelium. Scattered in the mesenchymal mass multiple islands of hyaline cartilage are present. Some strands of osteoid tissue are also seen, usually imperfectly surrounding the cartilaginous islands. Concentric arrangement of flattered spindle cells around cartilage shows definite transition to undifferentiated mesenchyme of the remaining parts, consisting of loose syncytium of stellated cells. Osteoid ground substance is deposited between those stellated cell in a shape of strands. Mitotic figures are fairly frequent. There is no cellular atypism (Fig. 3).

Boundary between the epithelial and mesenchymal elements is clear cut, and although imperfect, some basement membrane-like structures are seen. Direct contact or transition of the epithelium and cartilage has never been found. Both epithelial mucin and cartilaginous ground substance show positive mucicarmine stain, and metachromasia to toluidin blue at pH 3.6. McManus PAS stain reveals definite difference between the two.

(B) R-35-9: An irregularly ovoid, soft and grey-white mass of 5.0 cm diameter occupies the 5th lobe of the liver. It is mainly made up of fairly well

<table>
<thead>
<tr>
<th>D</th>
<th>R-37</th>
<th>4th</th>
<th>5.0</th>
<th>Cholangioma Muc. (#)</th>
<th>-</th>
<th>+</th>
<th>Cellular</th>
<th>175</th>
<th>592</th>
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<td>E</td>
<td>R-33</td>
<td>5th</td>
<td>Solid hepa. glandular</td>
<td>-</td>
<td>+</td>
<td>Cellular</td>
<td>166</td>
<td>566</td>
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<td>F</td>
<td>C-12</td>
<td>4th</td>
<td>trabecular hepatoma Glandular Cholangio. ca.</td>
<td>-</td>
<td>+</td>
<td>not very cellular, stellate</td>
<td>378</td>
<td>832</td>
<td></td>
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<td>G</td>
<td>C-13</td>
<td>2nd</td>
<td>Solid hepatoma Trab. hepa. glandular</td>
<td>-</td>
<td>+</td>
<td>Cellular</td>
<td>387</td>
<td>832</td>
<td></td>
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<tr>
<td>H</td>
<td>R-38</td>
<td>4th</td>
<td>Solid hepatoma glandular</td>
<td>-</td>
<td>-</td>
<td>(Ca-Sa.)</td>
<td>178</td>
<td>590</td>
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differentiated adenocarcinoma of cholangiomatous type. Some parts are solid, but marked honeycombed structures prevail in the other parts. Stromal tissues are unique. It consists of an edematous loose mass of well differentiated stellate cells with pale eosinophilic cytoplasms. Spindle cells are rarely encountered. Capillaries are notably distended. A few islands of hyalin cartilage are seen scattered in this mesenchymal mass. Owing to paucity in cellularity of the loose connective tissue, such cartilaginous tissue appears to exist without transitionary zones. Some islands come almost in contact with cancerous tubules (Fig. 1).

In contrast to the previous case, here we see frequently destruction of carcinomatous tubules, which spill out mucinous content into the subjacent mesenchyme. Transition between cartilaginous ground substance and spilled mucin has never been seen.

(C) R-28-3: The 2nd lobe had 4.5×4.0×2.5 cm ovoid grey-white mass, which is made up mainly of carcinomas, a cholangiocarcinoma and an adenohepatoma apparently colliding upon each other. Advanced cholangiofibrosis is present in close relation to the cholangiocarcinoma. Stroma of the adenohepatoma is narrow and not remarkable, while that of the cholangiocarcinoma is fairly broad and consists of characteristic structures. Fibers directly subjacent to the cholangiocarcinomatous epithelium are definitely collagenous with long sharp spindle cells. The cores of the stroma are loose stellate cells with multiple tiny rounded masses of hyaline cartilage. Transitions between the collagenous part and loose stellate mass, and between the latter and cartilaginous islands, are obvious. Some carcinomatous tubules are distended, some are collapsed and degenerated, with their mucinous contents spilling out into the subjacent mesenchymal mass. No reactive response or organizing process is seen. Cartilages are quite independent from this spilled mucin (Fig. 2).

(D) R-37-2: A soft grey-white mass of 5.0×6.0×4.0 cm, with occasional hemorrhagic–necrotic foci, occupied almost the entire 4th liver lobe. It consists of cholangioma with remarkably distended glandular structures, which spill out mucinous mass into the subjacent stroma. In the rather broad sheats of loose stellate cell mesenchyme, many large islands of hyaline cartilage are scattered. High cellularity of the undifferentiated mesenchyme and well differentiated cartilaginous structures are quite similar to those in the first example (Fig. 5).

(E) R-33: A small (0.5 cm), rather firm, grey-white mass from the 5th lobe, which was resected on the 166th day of experiment for serial transplantation, consists of a solid hepatoma with partly dilated glandular structures. Stromas are narrow and non-remarkable, except a few sparsely scattered, minute cartilaginous nodules. Although not fully differentiated, they have every characteris-
tic of hyaline cartilage with metachromatic hemogenous ground substance embedding ballooned chondros. Usual young mesenchymal masses, intervening between those nodules and carcinomatous epithelium are almost entirely lacking. The nodules often appear as if they were inserted into the narrow strands of stroma artificially. Spilling out of mucin is not noted.

(E') The second generation of the tumor E, submitted to a serial transplantation to peritoneal cavity of stock rats, showed, on the 14th day of transplantation, similar finding as in E. Only the cartilaginous masses are somewhat larger than those observed in the original tumor. Stellate mesenchymal tissues are just noticeable between the hyaline cartilages and carcinomatous epithelium of a highly cellulated solid hepatoma showing no glandular structures in it. Some cartilaginous masses have apparently undergone degeneration, granulated, basophilic and necrobiotic (Fig. 4).

Further serial transplants up to the 30th generation have so far revealed no such cartilaginous mass.

(F) C-12-Z 4: The 4th lobe contained a trabecular hepatoma with glandular structures and a cholanginoma associated with cholangiofibrosis. A few small islands of hyaline cartilage were seen scattered in broad sheet of relatively mature stellate cell mesenchyme. General pattern is quite similar to that of (B) (Fig. 6).

(G) C-13-2: Multicystic dilated structures of cholangiocarcinoma, measuring 2.0×2.5×2.0cm in greatest dimensions, occupies the 2nd lobe. Hemorrhagic necrosis has occurred rather extensively. Only a single minute island of cartilage has been found in step sections. Undifferentiated mesenchyme resembles with that of the previous case.

(H) R-38: The 4th lobe is entirely replaced by a solid hepatoma with a part of glandular structures at one side, where the interstitial tissues are remarkably broad and consist of dense sheets of middle sized stellate cells. High atypism, hyperchromatism and abundant mitosis of their nuclei, associated with polymorphism of cytoplasm, occasionally forming small giant cells, strongly suggest the stromal mass being malignant. No transition is observed between the carcinomatous epithelium and the stromal cells. Adjacent liver capsule is infiltrated only by the atypical stromal cells. (Fig. 7).

SUMMARY AND DISCUSSION

1. Types of mesenchymal proliferation above are as follows:
   a) Young edematous mesenchyme, consisting of loose mass of stellate cells or of fat spindle cells of non-atypical appearance, around cancerous alveoli or cartilage. It can be at times very highly cellular with crowded fat spindle cells, which have basophilic cytoplasm, but also can be made up of sparsely disseminated
rather acidophilic cells, anastomosing each other. Lymphocytes or other free cells are only very rarely seen (Fig. 1).

Of note is the sharp delineation of the proliferating immature mesenchyme against adult fibrous interstitium, composing normal liver parts, ordinary stroma of hepatic cancers or cholangiofibrosis.

b) Islands of hyaline cartilage, regularly surrounded by loose young mesenchyme above. Although they can be quite close to other structures, they are never seen exposed. They never show atypism, either of nuclei or cytoplasm. Their ground substance is that of regular hyaline cartilage (Fig. 1).

c) Fragments of osteoid tissue embedded in the young mesenchyme, and located in the midportion between an island of hyaline cartilage and a cancerous alveolus. They lack of any kind of malignant atypism, or of high proliferative activity. Calcification is very limited.

Such findings as indicate conversion of cartilage into osteoid or bone were never encountered. Membraneous character of the bone is apparent. Sometimes an arteriole is seen in the center or vicinity of osteoid formation.

d) High atypism of the mesenchymal elements, showing invasive growth into the hepatic capsule and diaphragm, and with suggested focal destruction of coexistent carcinoomatous tissue, was found only in the last example (Fig. 7 and 8).

2. Epithelial components and mesenchyme:

a) As has been stated the loose mesenchymal tissues are very intimate with the cancerous epithelium. They act precisely as a host stroma of the carcinoma, playing the role of primary stroma (Yoshida), which supplies both nourishment and stand point to the malignant epithelioma. They never appear to be invaded, lysed or amputated by the cancer. Cancer and mesenchyme make up an organoid structure. Necrosis and degenerative process are extremely rare. Degeneration of carcinomatous tubules or cystic glands appears to occur from other causes. Mucinous contents may be spilled out or left in the mesenchyme, without causing remarkable reactive change in the latter. Myxomatous transformation, as observed by Kinoshita, was unusual in our series. We could not confirm Kinoshita’s finding that the proliferated mesenchymal mass exerts pressure upon the parenchyma, either benign or malignant. Cartilaginous element is usually situated in the center of a mesenchymal stand, showing definite transition between the two kinds of tissue.

b) Pattern of the epithelial elements does not vary very much in relation to the mesenchymal reaction. Liver parenycyma, bile ducts, hepatic adenoma and cholangiofibrosis have constantly connective tissues of adult type as their interstitial tissues.

Carcinomas associated with this type of mesenchymal proliferation are pre-
dominantly cholangiocarcinoma with cyst-like dilated spaces or hepatoma with
glandular pattern. Close relationship between the carcinomatous epithelium
which lines glandular spaces and the mesenchymal reaction is of note. Many
of such tumor types are associated with cholangiofibrosis. It is also note-worthy
that focal squamous metaplasia was seen several times in the atypical columnar
epithelium adjacent to islands of cartilage (Figs. 4, 5 and 6).

Kinoshita (1937) describes in detail proliferation of connective tissues in the
early histogenesis of cholangioma. Sometimes, in his experimental series,
proliferation of stellated cells were so active that, in association with degenera-
tive changes on the part of epithelial elements, myxomatous structures occurred. He also got a “hepatoma combined with reticulosarcoma.” Fig. 57 of
Kinoshita almost exactly reduplicates our findings in No. A, D, E, G with
marked cellular proliferation of undifferentiated mesenchymal stellate cells.
Neither nuclear or cytoplasmic atypism is conspicuous.

Coordinate effects of DAB on parenchyma cell of the liver on one hand, and
on the stromal tissue, including endothelial cells, reticulum cells and fibroblasts
have already been pointed out, also by Kinoshita.

Incidence of cartilaginous structures was considerably high in our series, as
compared with that of the previous workers. 13.7% of 51 tumor bearing rats,
and 0.036% of 223 gross tumors found in the rat liver contained such structures.

A positive relationship between the kind of basic diet and incidence of hyper-
plastic mesenchymal reaction was not confirmed, although the group R (rice
diet) showed highest incidence of 14.7% and none of the group B (butter diet)
had cartilage containing tumor.

Chemical variation of carcinogenic amino-azo dyes appears so far to be of
little consequence in inducing mesenchymal reaction in discussion.

Richardson et al. stated that bone and cartilage appeared after 21 weeks of
experiment. In our present series the peculiar reactions were observed in the
rats kept for rather long period, ranging from 156 to 378 days.

Rats apparently tend to produce cartilage in liver under experimental condi-
tions. Kinoshita has got stellate cell proliferation in fowls, Andervont and
Dunn hemangioendothelioma in mice, and Nelson and Geoffrey (1953) some
mesenchymal proliferation in dogs after feeding amino-azo dye, but no cartilage.
Bullock and Curtis (1925) found four cartilage containing tumors among 1,400
rats infested with Cysticercus fasciolaris. Two of them contained hyaline
cartilage and two contained both cartilage and osteoid tissue or bone. They
were mixed cell sarcomas containing islands of cartilage, a chondrosarcoma, an
osteochondrosarcoma and an osteoid chondroma. Another case of cysticercus
tumor of rat, with a chondrosarcoma and a carcinosarcoma containing chondro-
matus components, was added by the same authors (1928). They used a strain
of Copenhaagen rats. Other stock rats have up to present produced (1951) cysticercus sarcoma rather infrequently.

Foulds (1937) reported a strain of transplantable oviduct carcinoma in domestic fowls which on serial transplantation produced bone and cartilage. His interpretation was that the carcinoma induced host stroma to form such structures. Undifferentiated mesenchymal reaction occurred always antecedent to such special structures. Andervont & Dunn's findings (1952) on serial transplantation of induced and spontaneous hepatomas in homologous mice was very remarkable. They saw hemangioendotheliomas and reticulum cell sarcomas arose from the peripheries of transplants. Hyperplasia of surrounding mesenchymal tissue appeared to precede such induced heterologous malignancies. Transplanted hepatoma disappeared as the second malignancy grew to take its place.

14 hepatic tumors in this series were excised by laparatomy from the primary hosts, and transplanted into other albino rats of the same stock, serially. Only 4 took in the first generation, of which one was followed up to the 30th generation. Cartilaginous mass was observed only in the second generation of transplantation and failed to reappear in the following generation to the end. The cartilage appeared more mature in the transplant than in the original liver tumor and somewhat degenerated, suggesting that it was not a autochthonous proliferation.

There have been many report since Apolant & Ehrlich (1906) concerning transformation of an animal carcinoma into a sarcoma during serial transplantation. Sanford et al. (1952) have obtained sarcoma by implantation of serially cultured carcinomas of liver, breast and thyroid. Tootian & Kidd (1950) saw a mammary carcinoma transforming into a sarcoma in an immunized animal. Such unexpected findings of transformation of epithelial tumors into mesenchymal ones, although they can be interpreted otherwise, might suggest epithelial character of the "mesenchymal" hyperplasia in appearance. However, in our experiments, thorough examination failed to reveal any transition of the carcinomatous elements and stromal hyperplasia.

Two possibilities are suggested for the causation of this peculiar mesenchymal proliferation with cartilaginous differentiation. This may be either a primary reaction of the hepatic interstitium to the carcinogenic substance, or a secondary induction in the stroma by the epithelial malignancy, first produced by the carcinogen. In view of rather rare occurrence, close histological relation to the epithelial components and long latency, the latter interpretation seems to be more acceptable.

On the other hand, knowledge has been accumulated concerning mixed tumor of human liver. Among many reports, only a few are acceptable as tridermal neoplasma, instead, majority of them appear to be more adequately explained.
on the basis of bidermal hyperplasia: hyperplasia of entodermal and mesodermal components only. In many cases, epithelial hyperplasia is far predominant over the mesenchymal one, which may actually be due to secondary induction exerted by the epithelial tumor (Someya, 1951, directed by one of the authors).

**RESUMÉ**

1. Incidence of peculiar mesenchymal hyperplasia with formation of cartilage was 13.7% of all tumor bearing rats fed with DAB.
2. Histological details were described.
3. Its histogenesis was discussed. Theory of secondary induction by DAB induced cholangiocarcinoma and glandular hepatoma was favored in its causation.
4. Histogenesis of mixed tumor in human liver may be interpreted also in this way.

**REFERENCES**

要　旨

癌原性アゾ色素によるネズミ肝の間葉性反応、特に軟骨形成及び肝の混合腫瘍の発生について

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木下（1937）以来、dimethyl-aminoazo-benzene ネズミ肝癌実験において、間葉性反応の著しいことが注目されてきたが、これについての詳細な記載は不足していた。著者らは、および高脂肪食を基本食餌として行った同実験について、間葉性反応を分析記載し、ことに軟骨の出現と、間葉性反応一般並びに癌腫上皮成分との相関関係について論じた。軟骨島は肝癌51例中8例において見られ、一例では移植一代に現われた。また一例の癌肉腫についても記載した。すべてに上記の例に著しい間葉性増殖が認められ、幼若型から種々の段階の分化をも追求できた。軟骨島が、腺管形成のある癌組織に直接する関質に認め得られたことは、癌上皮の間葉分化誘導を示唆すると思われる。
Fig. 1. (R 35-9) The edematous mesenchyme with stellate cells. Note paucity in cellular population. Cartilage is seen in the center.

Fig. 2. (R 28) A young cartilage surrounded by fat spindle cells. On the right carcinomatous glands with mucinous secretion. Note that there is no transition between the cartilage and carcinomatous epithelium.

Fig. 3. (R 39-Z) Cartilage in a dense population of proliferating mesenchyme.
Fig. 4. (R-33E'-Transplant) Cartilage found in a second generation transplant of a cartilage containing hepatoma. Degenerative changes are present. 14th transplantation day.

Fig. 5. (R 37-2 b) Relationship between glandular structures of the carcinoma and cartilage.

Fig. 6. (C 12-Z4) An abrupt appearance of a cartilaginous islet in parenchymatous carcinoma. Surrounding mesenchyme is remarkably thin.
Fig. 7. (R 38-3) Sarcomatous proliferation of the mesenchymal components. On far right and below degenerated hepatoma cells.

Fig. 8. (R 38-3) Detail of cellular pattern in Fig. 7. Note marked atypism and polymorphism.

Fig. 9. (R 22-1) Cholangiofibrosis, mature connective tissue in this picture is quite different from the peculiar immature mesenchyme in Fig. 1–6.