Increase of Extravascular Albumin Pool and the Intracellular Accumulation of Vesicles in Transplanted Morris Hepatoma 9121

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SUMMARY

Biosynthetically labeled albumin-3H from donor rats was injected into the femoral vein of 18 tumor-bearing rats. Blood samples, hepatomas (two per animal), and livers were removed at 10, 25, or 40 min after injection. Albumin, protein, and radioactivity were measured and albumin pool sizes were calculated. The values obtained for 10, 25, or 40 min after injection did not differ significantly. This demonstrates that the injected albumin-3H was distributed homogeneously in the blood stream throughout the body, including liver and hepatomas, and that no detectable change of the distribution of albumin between intravascular and extravascular compartments occurred during the experiment. The mean vascular albumin content of host liver was 2.1 ± 0.1 mg/g, wet weight, and that of Morris hepatoma 9121 was 1.4 ± 0.1 mg/g, wet weight. The mean extravascular albumin content of the hepatoma was considerably larger (3.5 ± 0.1 mg/g, wet weight) than that of host liver (1.0 ± 0.1 mg/g, wet weight). The ratio of extravascular albumin to total soluble protein was about 6 times larger in the hepatoma than in host liver. These values were independent of tumor size (weight range, 3.6 to 10.1 g).

Eighteen of the Morris hepatomas 9121 with weights ranging from 0.6 to 14.5 g were examined by light microscopy, the proportion of solid tumor tissue to tubular and necrotic areas decreased with increasing tumor weight. The fine structure of the hepatoma cells differed from the appearance of a hepatocyte most conspicuously by the accumulation of clustered vesicles. Well-developed Golgi complexes with many associated secretory vesicles were more numerous than in liver parenchyma. The rough endoplasmic reticulum was less frequent than in liver and usually formed slender and isolated cisternae, characteristic of minimal-deviation hepatomas. Free polyosomes appeared to predominate over bound ones. Microvilli-like structures were rare.

INTRODUCTION

In contrast to normal liver, transplanted hepatomas Morris 5123tc and 9121 and Reuber H35tc do not secrete protein. In the ultrastructural studies on Morris hepatoma 9121 reported in this paper, we examined the intracellular organelles probably involved in the synthesis and secretion of serum protein. A conspicuous accumulation of clustered vesicles within many tumor cells is described. The possible correlation between secretory defect, albumin accumulation, and appearance of intracellular clusters of vesicles is discussed.

MATERIALS AND METHODS

Animals and Hepatomas. ACI rats were maintained under conditions described previously (39). The Morris hepatoma 9121 was provided in generation 19 by Dr. H. P. Morris, Washington, D.C., and has been retransplanted in our laboratory as described previously (39). The experiments reported in this paper were performed on tumors of generation 54 at 21 days after transplantation. Tumors had been transplanted i.m. into both hind legs of the rats.

Injection of Albumin-3 H and Preparation of Tissues. Rats were maintained under light ether anesthesia for injections and
operations. For avoidance of the effect of chemical alteration of the albumin molecule on its distribution and metabolism in the body (5, 11, 19, 49), only freshly prepared, biosynthetically labeled albumin was used. For labeling of albumin, 32 normal male rats were given injections of 2 mCi of L-leucine-4,5-3H, 1 Ci/m mole (Radiochemical Centre, Amersham, Great Britain) per animal. Radiochemically pure albumin-3H was isolated according to the method of Schreiber et al. (46). The obtained albumin-3H (specific radioactivity, 0.03 µCi/mg) was injected into the right femoral vein of 18 male rats with an average body weight of 242 g, bearing Morris hepatoma 9121. Per 100 g of body weight, each rat received 0.137 µCi albumin-3H (1.33 mg) in 0.18 ml of 0.9% sodium chloride solution. The 18 tumor-bearing rats were divided into 3 groups of 6 animals each. One ml of blood per rat, tumors, and liver were removed at 10 to 11 min after injection of albumin-3H in the 1st group, at 25 to 26 min after injection in the 2nd group, and at 40 to 41 min after injection in the 3rd group. Care was taken that most of the blood was retained in tumors and livers. Each blood sample was mixed immediately with 1 ml of 0.9% sodium chloride solution. Tumors and livers were weighed after excision and stored at -20°.

**Extraction of Protein.** Frozen tissues were minced with scissors and homogenized with an Ultra-Turrax homogenizer (Janke und Kunkel, Staufen, Germany) in 10 volumes of ice-cold 0.2 M Tris-HCl, pH 7.6, containing 0.7% deoxycholate. Extensive homogenization and treatment with deoxycholate is the method assumed to result in the complete extraction of total albumin from the tissue (8, 20, 31). Homogenates were centrifuged for 90 min at 20,000 X g. The resulting pellets were homogenized again with 5 volumes of the above solution and centrifuged. The supernatants of both centrifugations were combined. In these extracts protein concentrations were measured by the biuret method (4) with bovine serum albumin as standard. The immunological procedure of Mancini et al. (27) with rat serum albumin as standard was used for the quantitative determination of albumin. The preparation of antisera is published elsewhere (44). The obtained data were used to calculate the total protein and albumin pool in the tissues.

**Determination of Radioactivity in Serum and Tissue Extracts.** Albumin was measured in an aliquot of serum. Another aliquot of serum was mixed with 1 M Hyamine hydroxide in methanol, incubated for 12 hr at 37°, and counted in ANPO (a scintillation counting fluid composed of 295.2 g naphthalene, 18.4 g PPO, 0.1839 g a-naphtholphenyloxazole, 1400 ml xylene, 1400 ml dioxane, and 840 ml absolute ethanol) in a Nuclear-Chicago Mark I liquid scintillation counter. Toluene-3H (1.84 X 10⁶ dpm/ml at the time of the experiments) (New England Nuclear, Boston, Mass.) was used as internal standard to calculate the absolute counting efficiency.

Before measuring radioactivity in the tissue extracts, extracts were concentrated by diafiltration in the Amicon ultrafiltration equipment with a UM20E filter (Amicon N.V., Oosterhout, Holland). Resulting precipitates were removed from the concentrated extracts by centrifugation. Pellets were discarded and an aliquot of the clear supernatant was used for measuring albumin again. Another aliquot of this supernatant was treated in the same way as the serum for the determination of radioactivity. These data were used to calculate specific radioactivity of albumin.

**Light Microscopy.** Portions of the tumor were fixed in buffered (pH 7.0) 4% formalin, dehydrated by graded ethanol solutions, and embedded in paraffin. Sections were stained with hematoxylin and eosin or with periodic acid-Schiff without or after diastase treatment.

**Electron Microscopy.** Small pieces of freshly removed tissue were fixed with 0.05 M cacodylate-buffered (pH 7.0) 2% glutaraldehyde for 30 min, postfixed with 2% OsO₄ for 2 hr, stained with 0.5% uranyl acetate for 8 hr, dehydrated with the use of graded ethanol solutions, and embedded in Epon. Thin sections were made with a Reichert OmU₂ ultramicrotome and double stained with uranyl acetate and lead citrate. Electron micrographs were taken with a Siemens-Elmiskop IA electron microscope.

**RESULTS AND DISCUSSION**

**Albumin Pools.** Albumin-3H was injected into the femoral vein of 18 rats bearing Morris hepatoma 9121. Blood samples and tissues were removed at 10 min (Group 1), 25 min (Group 2), or 40 min (Group 3) after injection. Protein was extracted from the tissues. Protein, albumin, and radioactivity were determined. Specific radioactivities of albumin in serum, host liver, and hepatoma as well as total pools of protein and albumin in both tissues were calculated. The obtained values are given in Table 1. Multiplication of total tissue albumin (A) by the ratio of the specific radioactivity of albumin from tissue (b) over that from serum (c) gives the vascular albumin content (V) of the examined tissue:

\[ V = A \frac{b}{c} \]

Subtraction of this value from total tissue albumin gives the extravascular albumin content (E) of that tissue:

\[ E = A - V \]

These values are shown in Table 2. Averaged from all 3 groups, the vascular albumin content of host liver was 2.1 ± 0.1 mg/g, wet weight, that of Morris hepatoma 9121 was 1.4 ± 0.1 mg/g, wet weight. The averaged extravascular albumin content of the hepatoma was considerably larger (3.5 ± 0.1 mg/g, wet weight) than that of host liver (1.0 ± 0.1 mg/g, wet weight). In host liver, extravascular albumin was 0.5% of total soluble protein, whereas in the hepatoma extravascular albumin amounted to 3.2% of total soluble protein. Our value for the extravascular albumin pool in liver concurs with the values reported by Peters (31). Peters found an intracellular albumin content of 0.88 mg/g liver, wet weight. He suggested that a small amount of this albumin may have originated from the interstitial space. No data are available for a similar evaluation of the distribution of extravascular albumin within the hepatoma, but the electron microscopic observations show that interstitial spaces in the tumor are much less developed than in liver.
Albunin Content and Fine Structure of Hepatoma

Table 1
Specific radioactivities of albumin in serum and in extracts from host liver and Morris hepatoma 9121 at different times after i.v. injection of albumin-3H

Per 100 g of body weight, each of the 18 rats bearing hepatomas received 0.137 µCi albumin-3H. Rats were then divided into 3 groups of 6 animals each. Blood samples, tumors, and livers were removed at 10 min (Group 1), 25 min (Group 2), or 40 min (Group 3) after injection of albumin-3H. Protein, albumin, and radioactivities were determined. Values are given as the mean ± S.E. Number of sera, hepatomas, and host livers are given in parentheses.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body wt (g)</th>
<th>Tissue wet wt (g)</th>
<th>Total protein (20,000 × g supernatant)/g tissue, wet wt (mg)</th>
<th>Total albumin/g tissue, wet wt (mg)</th>
<th>Specific radioactivity of albumin (dpm/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Host liver (6)</td>
<td>Host liver (6)</td>
<td>Host liver (6)</td>
<td>Host liver (6)</td>
</tr>
<tr>
<td>1 (10 min)</td>
<td>247 ± 5</td>
<td>6.8 ± 0.4</td>
<td>114 ± 3</td>
<td>5.4 ± 0.2</td>
<td>9,389 ± 285</td>
</tr>
<tr>
<td>2 (25 min)</td>
<td>238 ± 7</td>
<td>6.2 ± 0.6</td>
<td>108 ± 3</td>
<td>4.8 ± 0.2</td>
<td>9,437 ± 390</td>
</tr>
<tr>
<td>3 (40 min)</td>
<td>242 ± 3</td>
<td>7.0 ± 0.7</td>
<td>110 ± 3</td>
<td>4.5 ± 0.2</td>
<td>8,948 ± 364</td>
</tr>
</tbody>
</table>

Table 2
Vascular and extravascular albumin content of Morris hepatoma 9121 and host liver

From the data in Table 1, the vascular albumin content was obtained from multiplication of total tissue albumin by the specific radioactivity of albumin from tissue divided by the specific radioactivity of albumin from serum. Subtraction of the obtained value from total tissue albumin gives the extravascular albumin. Values are given as the mean ± S.E. of 6 livers or 12 hepatomas, respectively.

Albumin/g tissue, wet wt, in

<table>
<thead>
<tr>
<th>Time after injection of albumin-3H (min)</th>
<th>Vascular (mg)</th>
<th>Extravascular (mg)</th>
<th>Extravascular/total (%)</th>
<th>Vascular (mg)</th>
<th>Extravascular (mg)</th>
<th>Extravascular/total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 (Group 1)</td>
<td>1.8 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>35 ± 4</td>
<td>1.5 ± 0.1</td>
<td>3.9 ± 0.1</td>
<td>73 ± 3</td>
</tr>
<tr>
<td>25 (Group 2)</td>
<td>2.2 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>32 ± 4</td>
<td>1.3 ± 0.1</td>
<td>3.5 ± 0.1</td>
<td>73 ± 4</td>
</tr>
<tr>
<td>40 (Group 3)</td>
<td>2.3 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>27 ± 3</td>
<td>1.5 ± 0.1</td>
<td>3.0 ± 0.2</td>
<td>67 ± 5</td>
</tr>
</tbody>
</table>

In the experiments above, we removed blood samples and tissues at 10, 25, and 40 min after i.v. injection of albumin-3H. In the rat, i.v. injected compounds are distributed homogeneously throughout the blood within less than 2 min, as shown for 42KCl and 86RbCl (42), erythrocytes, and Evans blue (36). The data in this paper demonstrate also that in Morris hepatoma 9121 i.v.-injected albumin-3H was distributed homogeneously in the vascular compartment within less than 10 min, the vascular albumin pool being the same for 10, 25, and 40 min (Table 2). Compared to vascular distribution, degradation of albumin and its equilibration between the vascular and the extravascular compartment are very slow. More than 2 days were found to be necessary for the equilibration between vascular and extravascular pools after i.v. injection of plasma protein-3H (1), albumin-131I in rats (10, 25, 47) and rabbits (29), or of albumin-35C in rats (46). The half-life of total albumin is about 2.5 to 2.7 days (25, 46). Therefore, degradation and vascular-extravascular equilibration do not influence the data presented in Tables 1 and 2.

The preferential uptake and strong adsorption of i.v.-injected vital dyes by necrotic regions of tumors have been demonstrated in histological studies (18). Similar studies were reported with fluorescein-labeled albumin (13). In those experiments, the fluorescein-labeled albumin did not reach the necrotic areas. However, a strong albumin adsorption by dead cells was reported (13, 21, 40). Therefore, one might try to explain that the high extravascular albumin pool in Morris hepatoma 9121 is caused by albumin originating from the vascular compartment, penetrating into necrotic areas of this tumor, and thus being separated from the vascular space. Because of the random distribution of relatively small necrotic areas throughout Morris hepatoma 9121, the direct measurement of albumin concentration in excised necrotic portions is not feasible. W. B. Looney (personal communication) found in Morris hepatoma 9121 the proportion of necrotic areas in the total tumor increases with increasing tumor weight. Our own histological examinations on Morris hepatoma 9121 seem to confirm this observation. Thus, a correlation should exist between tumor size and concentration of albumin within the tumor if the above suggestion is correct. In Chart 1, albumin concentrations within the extracts are plotted against weight of tumors. The concentration of albumin within the tumor is independent of the tumor size, which suggests that the increase of extravascular albumin is not caused by a preferential accumulation of albumin within necrotic areas.

In the 20,000 × g supernatant, 111 ± 2 mg of protein were found per g of hepatoma, and 208 ± 6 mg/g of host liver. The average dry weight of the tumor (weight range, 6.6 g to 19.0 g) was 20.2 ± 0.3% (n = 7) of tissue, wet weight, while that of host liver was 27.8 ± 0.2% (n = 6) of tissue, wet weight. Thus, the water content of the hepatoma increased only slightly, excluding the presence of large edemas.

Light Microscopy. We examined 18 of the Morris hepatomas 9121 weighing between 0.6 and 14.5 g. All tumors showed the
histological characteristics previously reported by Hruban et al. (22) for Morris hepatoma 9121. In tumors of different size, however, we observed different proportions of solid tissue, tubular structures, and necrotic areas. In small tumors, the cells were arranged as a solid tissue sometimes containing small areas of necrosis. Only the central part of these tumors had a small area of trabecular organization. In the larger tumors, we observed extended areas of trabecular organization with large tissue gaps, numerous necrotic areas of different size, and only a thin periphery of solidly organized tumor tissue. Several tissue gaps were thin-walled vessels often containing erythrocytes. Several other tissue gaps of follicular structure seemed to be surrounded only by epithelia. No erythrocytes were observed in these gaps.

**Electron Microscopy.** The Morris hepatoma 9121 cells differ in appearance from a “normal” hepatocyte most conspicuously by the accumulation of vesicles in a great number of cells. The vesicles are usually clustered together in certain areas of the cell periphery (Figs. 1 and 2) and are sometimes extremely densely packed, so that often the membranes of adjacent vesicles come into direct contact (Fig. 2). Besides the predominant smaller vesicles (100 to 250 nm), larger ones are found which measure as much as 600 to 1000 nm in diameter. Sometimes these 2 vesicular size classes are arranged in such a way that the larger ones surround the smaller ones (Fig. 2). Such accumulation of vesicles resembles the apical pile-up of vesicles in the acinar cells (24) and also the situation in hypoxic liver cells as reported by Bassi and Bernelli-Zazzera (3). Most of the vesicles are surrounded by a smooth membrane whereas some, especially the larger ones, appear to be studded with ribosomal particles (Fig. 1). The vesicular content was relatively electron transparent. Well-developed Golgi complexes with many associated secretory vesicles are more numerous than in liver parenchyma (Figs. 3 and 4). Some of the Golgi vesicles contained an electron-opaque material. Coated vesicles are present in the vicinity of the Golgi complex (Figs. 3 and 4) and, in rare cases, they are also observed next to the plasma membrane (Fig. 1). The rough endoplasmic reticulum usually forms slender and isolated cisternae frequently filled with some fibrillar material. The cisternae either lie free in the cytoplasm or they encase parts of the mitochondrial surfaces. Many of the cisternae of the rough and the smooth endoplasmic reticulum are inflated. “Paired cisternae” and annulate lamellae associated with dictyosomes (28) (Fig. 3) are less frequently seen. “Free” polysomes predominate over membrane-bound ones, a situation which is generally regarded as a characteristic difference between a hepatoma cell and a hepatocyte (23, 34, 51). The intercellular space in the hepatoma is very small. The cell surfaces are predominantly parallel with a separation distance of about 15 to 80 nm. Microvilli-like structures or formations resembling bile-canaliculi (Fig. 1) are rare. Junctional complexes are frequent (Fig. 1). Vesicles protruding from the plasma membrane into the cytoplasm are occasionally found, but any decision as to whether this represents an endocytotic or exocytotic process is not possible.

Multivesicular bodies as well as typical lysosomes are usually present in the cell portions containing vesicles described above (Fig. 1). “Dark cells”, as shown for various other hepatomas (23), were also observed but may indicate necrotic stages rather than the existence of 2 different cell types within the tumor. Tangles of cytoplasmic microfilaments, as well as microtubules (Fig. 1) are frequently seen, especially in the portion of the cell that contains vesicles.

**Concluding Remarks.** The high extravascular albumin pool of the Morris hepatoma 9121 can be due either to an uptake of albumin from the vascular pool or to a retention of albumin synthesized by the tumor cells.

Concerning the uptake of albumin from the vascular into the extravascular space, we must distinguish between an uptake into the extravascular-extracellular, and an uptake into the intracellular compartment. As shown by electron microscopy, the interstitial space is very poorly developed and seems not to be sufficient for the storage of large amounts of albumin. However, many of the numerous tissue gaps seem to increase with increasing tumor size. However, no corresponding increased albumin concentration was observed (Chart 1). Thus, significant storage of albumin in the gaps is unlikely. As discussed above, the same reasoning applies to the possibility that the strong binding of albumin to damaged cells (13, 21, 40) may account for the high albumin content in the hepatoma.

Previously, there had been conflicting views as to whether tumor cells take up albumin without prior degradation (e.g., Refs. 7 and 9). Recently, it has been concluded from extensive studies that under physiological conditions only small amounts of albumin were taken up by tumor cells and that ingested albumin undergoes rapid degradation (cf. Ref. 41). Ryser (40) assumed that the initial observations on the high rate of plasma protein uptake by tumor tissues in vivo “... were due, in large part, to a pathological uptake by injured cells.” This kind of uptake “... appears to be associated with the formation of cytopathic uptake vacuoles, which are different from pinocytotic vesicles” (40). If, as Ryser suggests, the
injured cells are unable to hydrolyze rapidly the ingested protein, then the possible pathological uptake of albumin would account for the high albumin content in the Morris hepatoma as well as the observed intracellular accumulation of vesicles. We have no data to support this suggestion.

Concerning the possible retention of albumin synthesized by the tumor (39, 45), we have to consider 4 possible defects. There may be defects in (a) constitution of the membrane of endoplasmic reticulum-Golgi system, (b) constitution of plasma membrane, (c) conversion of albumin for transport through cell membranes, and (d) constitution of basement membranes around blood vessels.

Regarding the 1st possibility, Süss et al. (50) and Ragland et al. (35) investigated the interactions of polysomes and membranes of "striped" rough endoplasmic reticulum from rat liver and hepatoma in vitro. The capacity of membranes to bind polysomes was dependent on the source of rough endoplasmic reticulum membranes but independent of the source of polysomes. Süss et al. suggested that alterations of membrane structure may be responsible for this phenomenon. Regarding the 2nd possibility a possible alteration of plasma membrane structure is indicated by the findings of Reutter et al. (37), who reported a defect of UDP-N-acetyl-β-glucosamine-2-epimerase in Morris hepatomas 9121 and 5123tc. No data are available which give any indication to the last 2 possibilities.

Our findings on the decreased albumin synthesis (39, 45) and the lack of secretion (43) are not in agreement with the observations of Richardson et al. (38). These authors reported albumin synthesis and secretion in cell cultures of Morris hepatoma 7795. Further investigations are in progress in our laboratory in which a system of single-cell suspensions and animals to establish subcolonies at the Biochemisches Institut, Freiburg im Breisgau. We thank also Dr. W. W. Franke from the Biologisches Institut II and Dr. R. Reynolds from the Biochemisches Institut, Freiburg im Breisgau, for their helpful discussions.

ACKNOWLEDGMENTS

We are very grateful to Dr. H. P. Morris for providing us with tumors and animals to establish subcolonies at the Biochemisches Institut, Freiburg im Breisgau. We thank also Dr. W. W. Franke from the Biologisches Institut II and Dr. R. Reynolds from the Biochemisches Institut, Freiburg im Breisgau, for their helpful discussions.

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Figs. 1 to 4. Electron micrographs of Morris hepatoma 9121. M, mitochondria; N, nucleus; Fixation: glutaraldehyde, OsO₄, sequential use.
Fig. 1. Peripheral areas of 4 adjacent cells. Numerous vesicles (V) are next to the plasma membrane (PM). The intercellular space is very small. Vesicles protruding from the plasma membrane are rarely recognized (double arrow). Junctional complexes are frequent and often demarcate canaliculi-like spaces (BC), particularly seen in the inset. Multivesicular bodies (MV) are present. Short arrows, microtubules (MT); long arrows, coated vesicles. D, dictyosomes. × 25,600. Inset, × 21,000.
Fig. 2. Four adjacent cells. Smooth-surfaced vesicles (V) of 2 size classes are densely accumulated in the cell periphery. × 17,000.
Figs. 3 and 4. Well-developed dictyosomes (D) with associated secretory vesicles (V). In Fig. 3, a small annulate lamellar cisterna (AL) is in association with the dictyosome. PM, plasma membrane. Fig. 3, × 15,000; Fig. 4, × 19,000.
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