IN VITRO SYNTHESIS OF RAT SERUM LIPOPROTEINS AND PROTEINS BY MORRIS HEPATOMA 7777

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Received 31 July 1972

1. Introduction

We previously reported a large increase in the concentration of serum lipids and lipoproteins in Buffalo strain rats bearing transplanted Morris hepatoma 7777 [1]. This elevation in serum lipoproteins could have been contributed by the host liver, by the growing tumor or by both tissues. In an attempt to answer this question, we have investigated the incorporation of [U-^{14}C]L-leucine into five classes of serum lipoproteins by slices of normal liver, host liver and hepatoma 7777. There is very little information in the literature concerning the synthesis of serum lipoproteins by neoplastic tissues [2–4], and this investigation has demonstrated an increased capacity for serum lipoprotein and protein synthesis by Morris hepatoma 7777 as compared to both host liver and normal liver.

2. Methods

Control Buffalo-strain rats (female, ave. wt. 170 g) as well as rats bearing transplanted Morris hepatoma 7777 (generation 62) were supplied by Dr. H.P. Morris. Hepatoma 7777 is a rapidly growing, poorly differentiated tumor [5]. Five weeks after the injection of the tumor, blood was obtained through the abdominal aorta of normal and tumor-bearing rats under Nembutal anesthesia and the livers and tumors were quickly excised and chilled in Krebs–Ringer bicarbonate buffer held at 0°. Five normal and five tumor-bearing animals were used in the present experiments for the preparation of liver or tumor slices. The liver slices (0.5 mm thick) were rapidly prepared using a Stadie–Riggs microtome and slices from each liver or tumor were held separately in cold Krebs–Ringer-bicarbonate buffer maintained at 0°. In the case of tumor, care was taken to discard any necrotic areas; however, with this tumor, at this time period, necrotic areas were seldom seen. This tumor was fragile and did not slice well. Therefore, it was necessary to use several small pieces of slices rather than three to four large slices as in the case of normal and host-liver. Weighed amounts of liver or tumor slices (close to 440 mg) were incubated in 25 ml Erlenmeyer flasks with 5 μCi of [U-^{14}C]L-leucine (210 mCi/mmole, Volk Radiochemical Co, Calif.) and 1 mg each of 22 unlabeled amino acids including L-leucine in 5 ml of freshly isolated normal serum from Holtzman rats at 37° for 4 hr under 95% O_2–CO_2 atmosphere in a Dubnoff metabolic shaker, essentially according to the procedure of Radding et al. [6, 7]. In two other flasks, 5 ml of serum without liver slices were incubated with the same amount of label (5 μCi) and unlabeled amino acids in order to account for any non-specific binding of labeled leucine to serum lipoproteins and proteins. The procedure used to isolate the radioactively-labeled lipoproteins and proteins, removal of excess radioactive leucine, method of solubilization and counting have been described ear-

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North-Holland Publishing Company – Amsterdam

Table 1

Incorporation of [U-^{14}C]leucine into serum lipoproteins, serum proteins and tissue proteins by slices of Morris hepatoma 7777, host liver and control liver.

Description of tissue	Lipoprotein or protein	Total activity[a] (cpm/5 ml medium)	Specific activity (cpm/mg protein)
Normal liver		302 ± 70[b]	600 ± 126
Host liver	VLDL ($d < 1.019$)	440 ± 158	791 ± 247
Tumor		1779 ± 366 ($P < 0.001$[c], $P < 0.001$[d])	3113 ± 703 ($P < 0.005$, $P < 0.001$)
Normal liver		94 ± 23	134 ± 30
Host liver	LDL ($1.019 < d < 1.050$)	172 ± 48 ($P < 0.01$[c])	232 ± 56 ($P < 0.01$)
Tumor		635 ± 189 ($P < 0.005$, $P < 0.01$)	914 ± 217 ($P < 0.005$, $P < 0.005$)
Normal liver		174 ± 23	218 ± 27
Host liver	HDL_1 ($1.050 < d < 1.063$)	257 ± 63 ($P < 0.025$)	322 ± 75 ($P < 0.025$)
Tumor		987 ± 161 ($P < 0.001$, $P < 0.001$)	1136 ± 190 ($P < 0.001$, $P < 0.001$)
Normal liver		382 ± 80	130 ± 24
Host liver	HDL_2 ($1.063 < d < 1.125$)	887 ± 127 ($P < 0.001$)	300 ± 51 ($P < 0.001$)
Tumor		3022 ± 840 ($P < 0.005$, $P < 0.005$)	1090 ± 306 ($P < 0.005$, $P < 0.005$)
Normal liver		28 ± 29	29 ± 27
Host liver	HDL_3[e] ($1.125 < d < 1.21$)	158 ± 67 ($P < 0.005$)	156 ± 64 ($P < 0.005$)
Tumor		565 ± 207 ($P < 0.005$, $P < 0.005$)	596 ± 202 ($P < 0.005$, $P < 0.01$)
Normal liver		3846 ± 790	12 ± 3
Host liver	BFP[f] ($d > 1.21$)	18337 ± 8806 ($P < 0.025$)	56 ± 25 ($P < 0.025$)
Tumor		34217 ± 7408 ($P < 0.001$, $P < 0.025$)	110 ± 23 ($P < 0.001$, $P < 0.01$)
Normal liver			3408 ± 341
Host liver	Tissue protein		5670 ± 1514 ($P < 0.05$)
Tumor			27215 ± 4549 ($P < 0.001$, $P < 0.001$)

[a] The total activity given is the radioactivity released into 5 ml of medium in 4 hr.
[b] Mean ± S.D. of five separate determinations from liver or tumor from individual rats.
[c] Compared against control liver.
[d] Compared against host liver.
[e] The HDL_3 fractions were purified by a second ultracentrifugation at density 1.21.
[f] Bottom fraction proteins, i.e., total serum proteins devoid of most lipoproteins.

lier [8]. In brief, after removal of cellular debris by sedimentation at 28,620 *g* for 20 min, five classes of lipoproteins (very low density lipoproteins, VLDL; low density lipoproteins, LDL; high density lipoproteins, HDL_1, HDL_2 and HDL_3) were ultracentrifugally isolated at 10° using solution densities (1.019, 1.050, 1.063, 1.125 and 1.21 g/ml) at 114,480 *g* according to the technique of Havel et al. [9]. The lipoproteins and proteins were dialyzed exhaustively against physiological saline containing 0.05% unlabeled leucine, 0.01% merthiolate and 0.005% versene in order to remove free radioactive leucine. Aliquots of the dialyzed fractions were solubilized in NCS reagent and counted at approx. 80% efficiency in a liquid scintillation counter. By measuring the radioactivity of the lipid extracts of the various fractions, it was established that labeled leucine was almost entirely incorporated (between 90 to 95%) into the protein moiety of the lipoproteins. Protein was determined according to the procedure of Lowry et al. [10]. The results have been expressed both as total activity (cpm/5 ml medium) as well as specific activity (cpm/mg protein). Immediately after incubation, liver and tumor slices were rinsed quickly in ice cold saline, homogenized in cold saline and aliquots were solubilized and counted as described above. Portions of the homogenates were also rapidly treated with hot 80% ethanol in order to extract the free amino acids in the tissues. Aliquots of these extracts were also counted in a liquid scintillation spectrometer.

3. Results and discussion

The total incorporation of radioactive leucine into serum lipoproteins and total serum proteins was many fold greater with tumor slices as compared with normal liver slices. The host liver incorporated labeled leucine into serum proteins and most serum lipoproteins to a much greater extent than control liver but to a lesser degree than the tumor (table 1).

While the total incorporation of the label into VLDL by the host liver was not significantly different from that observed with normal liver, it was several fold increased with Morris hepatoma 7777 ($P<0.001$). The host liver and tumor slices, respectively, incorporated about 2 and 6 times as much of the label into LDL as compared with normal liver slices. In both cases, the difference between control liver and tumor or host liver was highly significant ($P<0.005$, $P<0.01$, respectively). In spite of the increased incorporation into LDL by the host liver, the tumor slice was significantly more active ($P<0.01$). With the lipoprotein of intermediate electrophoretic motility, HDL_1, the incorporation was also significantly greater with the tumor as compared with the control and host liver ($P<0.001$).

In the case of the principal lipoprotein (HDL_2) of rat serum, the total incorporation by the tumor was about 8 times of that incorporated by the normal liver ($P<0.005$). The host liver was also considerably active ($P<0.001$, host *vs.* control) but it incorporated only a third as much as the tumor ($P<0.005$). In view of the low counts observed with the HDL_3 faction from normal liver and also due to high variability of the results in this case, the absolute value for total incorporation may be subject to considerable error. However, it is noteworthy that the ratio of incorporation into this lipoprotein by host liver to that by the tumor paralleled the behavior observed with HDL_2.

Disc electrophoresis was performed on several samples from each lipoprotein class isolated from the medium (serum) that was used for the three systems (control liver, host liver and tumor). The amino black patterns were substantially similar to that reported earlier for lipoprotein fractions isolated from normal rat serum [11] and established the absence of any contaminating serum proteins in the various lipoproteins that were used in this study.

The protein synthetic capacity of both the tumor and the host liver was clearly reflected by the enormous increase (4–8-fold) in the total incorporation in total serum proteins (BFP) by these tissues as compared with normal liver ($P<0.001$, $P<0.025$, respectively). While the specific incorporation of the label into host liver was only about twice as much as into normal liver protein, it was increased 7 times in the case of the tumor proteins and appeared to reflect the rapid growth rate of hepatoma 7777.

The present results have demonstrated an enormous increase in the total incorporation of labeled leucine into serum lipoproteins and total serum proteins by hepatoma 7777 as compared with normal liver. These results confirm the data obtained in a previous *in vitro* investigation using hepatoma 7777, its host liver and normal liver conducted under the same con-

ditions described here except that unlabeled amino acids were not added to the medium. While the radioactivity was greater in all fractions, the trend in the results, particularly the ratios of incorporation between tumor and control were similar though not identical. In the present experiments, immediately after the incubation, the radioactive slices were rapidly homogenized (after quick rinses in cold saline), and treated with hot 80% alcohol to extract the free amino acids, which were then separated by chromatography. The specific radioactivity of free leucine thus isolated was approximately the same for the three tissues studied and suggested that the observed increase in radioactivity of the lipoprotein and proteins with tumor slices appeared to reflect true synthesis under defined optimum conditions and not merely elevated incorporation obtained fortuitously.

The present findings are also in agreement with the increased total incorporation reported earlier with a primary rat hepatoma induced by *N*-2-fluorenylacetamide [12]. As compared with the corresponding normal liver, the increased incorporation into LDL, HDL and BFP was several-fold greater with hepatoma 7777 than with the primary hepatoma, under the same experimental conditions. Since hepatoma 7777 is a well "characterized" tumor, it is possible that the increased incorporation may be related to the absence of normal and preneoplastic cells that may be expected to be present in primary hepatomas. On the other hand, the results may also be explained as due to the differences in characteristics between hepatoma 7777 and the primary hepatoma. While the primary hepatoma was histologically characterized as a well differentiated tumor of the trabecular type [13], hepatoma 7777 is a rapidly growing, poorly differentiated tumor [5]. Obviously, it would be of interest to investigate the incorporation of amino acids into serum lipoproteins and proteins using other Morris hepatomas, especially those which are medium or well differentiated and those which have a slower growth rate than hepatoma 7777. Although studies have been conducted on the incorporation of labeled amino acids into tumor proteins [14], surprisingly there is little information on the *in vitro* synthesis of serum proteins by Morris hepatomas of different growth rates. A correlation between the growth rate of the tumor and its ability to incorporate labeled amino acids into tissues proteins was reported by Wagle et al. [14]. It, however, remains to be established whether a similar relationship exists between the growth rate of tumors and the extent of synthesis of serum proteins and lipoproteins.

This study clearly showed increased incorporation into the protein moieties of LDL and HDL and was in contrast to that reported using cholesterol-fatty livers [7, 15]. In the latter case, the synthesis of the protein moiety was unaffected, whereas the secretion of cholesterol into the medium by fatty liver was increased as compared with normal liver. MaJerus et al. [16] have reported that, under certain conditions, hepatoma 7777 synthesizes fatty acids 7 times as fast as the host liver. Therefore, it appears likely that the lipid moiety as well as the protein moiety of serum lipoproteins are synthesized in quantity by hepatoma 7777.

Our earlier work has established that the presence of a large-sized hepatoma 7777 elicited a substantial increase in host serum LDL and HDL [1]. From the increased incorporation into several lipoprotein classes observed using host liver, it can be stated that the host liver contributed significantly to the increased level of lipoproteins in rats bearing hepatoma 7777. Whether the lipoproteins and proteins synthesized *in vivo* by the transplanted tumor were also released into circulation or were directly utilized by the tumor itself cannot be stated at this time.

The greatly elevated synthesis of serum lipoproteins and proteins by the tumor *in vitro* may represent a shunt mechanism because of the obvious lack of cellular growth per se under these conditions. Arguments have been previously advanced concerning the possible utilization of serum high density lipoproteins by dividing cells, perhaps in the assemblage of cellular membranes [11]. The greatly increased incorporation into BFP and the predominant lipoprotein of rat serum, namely HDL_2, may suggest utilization of these proteins in some manner by the tumor. In this context, it may be pointed out that Busch et al. [3, 17] have demonstrated that labeled albumin was incorporated to a much greater extent into tumor homogenates, mitochondria and microsomes than into the corresponding normal fractions. These and other studies have led to the suggestion that tumors make use of plasma proteins, particularly albumin, in the process of biosynthesis of their tissue proteins [3]. In what way the tumor is able to elicit a specific response in the host liver with respect to serum lipoprotein syn-

thesis and what changes within the host liver are responsible for the large increase in serum protein synthesis are fundamental questions that may have a bearing on the control of cancer.

4. Conclusions

The incorporation of [U-^{14}C] L-leucine into rat serum lipoproteins and proteins by rapidly growing Morris hepatoma 7777, its host liver and normal liver was investigated under *in vitro* conditions. The total incorporation into tissue proteins, serum proteins and lipoproteins was many fold greater with tumor slices as compared with normal liver slices and demonstrated that hepatoma 7777 has the ability to synthesize serum lipoproteins and proteins. The host liver incorporated radioactive leucine into serum proteins and most lipoproteins to a much greater extent than control liver but to a lesser degree than the tumor. The greatly increased incorporation, especially into serum proteins and the high density lipoproteins (HDL_2) by both host liver and tumor may suggest direct utilization of these proteins in some manner by the tumor.

Acknowledgements

This investigation was supported by research grants CA-01932 and CA-10727 and a research career development award, 5K3-CA-31,063 to K.A.N. from USPHS. Appreciation is due to Mrs. Linda Williams and Mrs. Linda Maynes for their valuable technical assistance.

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