Abnormal Distribution of O-Alkyl Groups in the Neutral Glycerolipids from Human Hepatocellular Carcinomas¹

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ABSTRACT

The direct comparison of O-alkylglycerol composition in the neutral lipids fractions prepared from human hepatocellular carcinomas with that in corresponding preparations from nonneoplastic liver is reported. Tumor-bearing liver and noncancerous liver specimens were obtained either during surgery or at autopsy. Thirty different tissue specimens obtained from 18 cases were analyzed. Representative samples from each specimen were examined microscopically to confirm the pathological diagnosis. Gas chromatographic analysis of alkylglycerol derivatives showed that hexadecylglycerol, octadecylglycerol, and octadecenylglycerol were the principal components. Compared to the noncancerous livers, hepatocellular carcinomas contained higher proportions of hexadecylglycerol and lower proportions of both C₁₀-glycerol ethers. Associated with this change was an increase in the proportion of saturated to monoenic alkylglycerols. These abnormalities appeared to be more severe in the necrotic areas of the tumors. Higher concentrations of neutral alkylglycerolipids and of cholesterol were found in the tumors; no differences between the two groups could be found in the levels of ether-linked phosphoglycerides, triglycerides, and lipid phosphorus.

INTRODUCTION

An increase in alklydiacylglycerols and a difference in the composition of glycosphingolipids have been mentioned as the 2 consistent features of the lipid composition of cancer cells (24). In particular, the rise in alklydiacylglycerols has been well documented (14). Although the compositions of the O-alky groups in tumor lipids have been reported (1, 8, 13, 15, 17, 18, 23), the question remains whether or not the observed distributions differ from those in the cells of origin (15). For an effective answer to this question, a comparison between nonaplastic and nonneoplastic elements of the same tissue is required. One technical drawback has been obtaining sufficient quantities of relatively pure samples of tumor and of nontumor tissue in which the cells giving rise to the tumor actually make up the major part of the tissue analyzed. We have been able to study this problem in human hepatocellular carcinomas because the incidence of primary liver cell tumors in Hong Kong is high (9). This type of tumor frequently forms a grossly definable mass with scanty stromal elements (3). The residual nonneoplastic portions of the tumor-bearing liver specimens and livers from other patients who had died of unrelated causes were used for comparison. Analysis by gas chromatography of highly purified alklyglycerols derived from the neutral lipids fractions showed that the composition in the tumors was abnormal; the key finding was an increase in the proportion of hexadecylglycerol.

MATERIALS AND METHODS

Tissue Specimens. The 9 specimens of noncancerous liver (Group C in Tables 1 and 2) were obtained at autopsy from 1 case each of bronchogenic carcinoma and carcinoma of the esophagus (both without liver metastases), 1 case each of bleeding gastric ulcer, myocardial infarction, chronic bronchitis with emphysema, diabetes mellitus, calcific aortic stenosis with heart failure, cerebral infarction, and chronic glomerulonephritis. Liver specimens with hepatocellular carcinoma were removed surgically by partial hepatectomy (4 cases) or at autopsy (5 cases). Surgical specimens were chilled immediately and processed within 1 hr. The tumor-free liver specimens and livers with other types of tumors were obtained at autopsy usually within 48 hr of death. Tumor-bearing liver was dissected into 2 portions, tumor and residual tissue, on the basis of naked-eye examination. In 2 hepatectomy specimens the tumor showed grossly necrotic areas that were further separated from nonnecrotic tumor and were also analyzed. Weighed tissue specimens (35 to 110 g) were stored in chloroform:methanol (2:1) at −20°C. Lipid extraction (5) was usually carried out within 2 days. Specimens known to be positive for hepatitis B surface antigen (3 cases) were immersed for 24 to 44 hr in 10% neutral-buffered formalin prior to lipid extraction. In a separate experiment it was found that this step had no effect on the distribution of alklyglycerols.

Tissue Histology. Representative blocks of tumor, adjacent nonneoplastic liver, and tumor-free livers were processed by standard histological techniques for the preparation of paraffin sections. The final grouping of the specimens for analyses of the results was based on the microscopic findings in these sections. The 9 specimens of hepatocellular carcinomas included trabecular, acinar, and anaplastic types; however, a mixture of these different patterns was often found in the same tumor, and no attempt was made to further subdivide this group. The amount of tumor necrosis was variable, but it was always present to some degree. In 1 of the 2 tumors that could be divided into necrotic and "nonnecrotic" areas on gross examination, microscopy showed small foci of necrosis even in the nonnecrotic portion.

The specimens of residual liver often showed microscopic foci of tumor in the portal venous radicles or within

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the regeneration nodules of cirrhotic livers, even when this was not obvious grossly. Such cases (6 specimens) were grouped and designated Residual Liver A in the tables. Only those cases in which microscopic examination of the residual liver showed no tumor cells were grouped as Residual Liver B (3 specimens).

The Group C referred to in the tables consisted of noncancerous liver specimens that showed varied degrees of congestion, centrilobular necrosis, and bile stasis related to the primary disease processes.

Two specimens of metastatic tumor deposited in the liver from a demonstrable primary in the bronchus were identified histologically as poorly differentiated adenocarcinoma and oat cell carcinoma. One primary liver carcinoma was found to have features more consistent with bile duct origin, with some squamoid differentiation. These 3 specimens were also analyzed.

Isolation and Analysis of Alkylglycerols. Neutral lipids isolated from the lipid extract with the aid of silicic acid column chromatography were subjected to saponification, and the alkylglycerols were recovered from the hydrolysate by extraction with chloroform (10). The alkylglycerols were purified by means of at least 4 cycles of thin-layer chromatography, elution and rechromatography; the developing solvents used in the different chromatographic separations were diethyl ether:acetic acid, diethyl ether:ammonia, and chloroform:butyl alcohol (9), in that order. The methods of derivative formation and the identification and quantitation of the individual alkylglycerols by gas chromatography of their trimethylsilyl and diacetyl derivatives were carried out according to procedures fully described elsewhere (10).

RESULTS

Table 1 gives the comparison of lipid composition in hepatocellular carcinomas to that in the residual tissues or in the noncancerous liver specimens. No difference was found between the surgical and autopsy specimens of tumor-bearing liver in the lipid components listed. The levels of neutral alkyl glycerolipids (which include alkylalcoholglycerols) were on the average higher in the tumors than in the tumor-free residual tissue or in noncancerous livers. The tumor specimens were in this respect more variable; the concentrations of neutral alkyl glycerolipids ranged from 0.5 to 18.7 µg/g. These results extend the observations of Snyder and Wood (19) who showed that on the whole different tumors in humans contain greater amounts of neutral O-alkylglycerols than do most normal tissues. The concentration of neutral alkyl glycerolipids in residual tissue infiltrated with tumor cells fell between those found in the tumors and in tumor-free tissue. Total cholesterol levels in hepatocellular carcinomas were also abnormally high. Elevated cholesterol levels have been reported in Morris hepatomas (17) and in plasma membranes isolated from mouse and rat hepatomas (25). The concentrations of other lipid components, including ether-linked phosphoglycerides, did not differ in the 4 groups. However, the levels of alk-1-enyl phosphoglycerides in the tumor specimens covered a notably wide range of 0.4 to 18.2 µg/g in the 2 autopsy specimens, with the concentrations in the hepatotomy specimens in between. No attempt was made to quantitate the neutral alk-1-enyl glycerolipids, which appeared to be in extremely low concentrations in the few preparations examined.

The composition of the O-alkyl moieties in the neutral glycerolipids of tumor tissue compared to that in noncancerous liver and in the residual tissues is summarized in Table 2. Hexadecylglycerol, octadecylglycerol, and octadeccenyglycerol (represented in Table 2 as 16:0, 18:0, and 18:1, respectively) were the principal components found. The composition of the tumor lipids was different in several respects from that in noncancerous liver (Group C). In the latter, hexadecglycerol, octadecylglycerol, and octadeccenyglycerol were present in ratios of nearly 1:2:2. In

Table 1

<table>
<thead>
<tr>
<th>Lipid composition of tissue specimens</th>
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<tbody>
<tr>
<td>Values were obtained on 9 noncancerous liver specimens (Group C) and 9 specimens of liver bearing hepatocellular carcinoma. Each of the tumor-bearing specimens was dissected into 2 portions, tumor and residual tissue. Based on histological examination, the latter was classified Group A (tissue containing tumor cells, 6 specimens) or Group B (tissue free of tumor cells, 3 specimens). The values for ether-linked glycerolipids (which were based on the molar absorbivities of alkylglycerol or alk-1-enylglycerol treated with acid and with fuchsin) are expressed as µg octadecyglycerol or octadecenylglycerol per g (wet weight) of tissue; the other values are in mg/g. The methods for the estimation of glyceryl ethers and of cholesterol have been given (10). Triglycerides were determined by the Hantzsch condensation reaction.</td>
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<table>
<thead>
<tr>
<th></th>
<th>Hepatocellular carcinomas (A)</th>
<th>Residual liver (B)</th>
<th>Noncancerous liver (C)</th>
<th>Tumor vs. A&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tumor vs. B&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Tumor vs. C&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral alkyl glycerolipids</td>
<td>3.5 ± 5.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.8 ± 0.9</td>
<td>0.1 ± 0.1</td>
<td>0.3 ± 0.4</td>
<td>NS&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Alkyl phosphoglycerides</td>
<td>4.9 ± 3.0</td>
<td>2.5 ± 2.0</td>
<td>4.7 ± 1.6</td>
<td>2.8 ± 2.6</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Alk-1-enyl phosphoglycerides&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.4 ± 6.9</td>
<td>2.3 ± 2.0</td>
<td>6.5 ± 0.1</td>
<td>3.8 ± 5.1</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>4.5 ± 2.4</td>
<td>2.6 ± 0.7</td>
<td>2.2 ± 0.8</td>
<td>1.7 ± 0.6</td>
<td>NS</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>4.0 ± 3.5</td>
<td>2.9 ± 2.3</td>
<td>0.9 ± 1.0</td>
<td>3.1 ± 3.1</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>12 ± 5.3</td>
<td>12 ± 6.7</td>
<td>12 ± 6.7</td>
<td>11 ± 3.6</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Total lipids</td>
<td>26 ± 10</td>
<td>22 ± 5.2</td>
<td>22 ± 6.1</td>
<td>19 ± 5.9</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
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<sup>a</sup> Student's t test for paired differences.
<sup>b</sup> Variance ratio or t test.
<sup>c</sup> Mean ± S.D.
<sup>d</sup> NS, not significant.
<sup>e</sup> Values obtained on formalin-fixed tissues, which are significantly lower than those in unfixed specimens, were omitted.
The specimens of tumor, residual liver containing tumor cells (Group A), tumor-free residual liver (Group B), and noncancerous liver specimens (Group C) are the same as those referred to in Table 1. Alkylglycerols with C16 and C18 groups amounted to 94 to 100% of all the components found in all except 1 of the specimens; 16% of nonadecenylglycerol was present in 1 tumor preparation. In a few other specimens, alkylglycerols with C16, C18, C20, and C22 groups were also found, with no bias toward tumor or liver preparations. The percentage composition was obtained by computing the peak areas in gas chromatograms of the trimethylsilyl derivatives.

<table>
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<th>Side chain</th>
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<td>16:0 (%)</td>
<td>46.8 ± 18.0d</td>
<td>26.0 ± 8.6</td>
<td>26.0 ± 16</td>
<td>21.7 ± 6.9</td>
<td>&lt;0.03</td>
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<td>18:0 (%)</td>
<td>26.0 ± 10.9</td>
<td>33.4 ± 6.9</td>
<td>38.0 ± 9.5</td>
<td>39.9 ± 5.9</td>
<td>NSf</td>
<td>NS</td>
<td>&lt;0.005</td>
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<td>18:1 (%)</td>
<td>23.4 ± 8.8</td>
<td>37.4 ± 11</td>
<td>36.3 ± 13</td>
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<td>C16:C18</td>
<td>1.72 ± 2.74</td>
<td>0.41 ± 0.17</td>
<td>0.40 ± 0.35</td>
<td>0.29 ± 0.13</td>
<td>&lt;0.05</td>
<td>NS</td>
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</tr>
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<td>Saturates:</td>
<td>3.52 ± 2.36</td>
<td>1.84 ± 0.79</td>
<td>1.95 ± 0.90</td>
<td>1.60 ± 0.45</td>
<td>&lt;0.05</td>
<td>NS</td>
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Table 2
Abnormal distribution of alkylglycerol moieties in the neutral lipids from hepatocellular carcinomas

The specimens of tumor, residual liver containing tumor cells (Group A), tumor-free residual liver (Group B), and noncancerous liver specimens (Group C) are the same as those referred to in Table 1. Alkylglycerols with C16 and C18 groups amounted to 94 to 100% of all the components found in all except 1 of the specimens; 16% of nonadecenylglycerol was present in 1 tumor preparation. In a few other specimens, alkylglycerols with C16, C18, C20, and C22 groups were also found, with no bias toward tumor or liver preparations. The percentage composition was obtained by computing the peak areas in gas chromatograms of the trimethylsilyl derivatives.

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DISCUSSION
The accumulation of glycerol ether lipids in neoplastic tissues may be the result of active synthesis within the tumor, in addition to the near absence of ether-cleaving enzymes (20, 21). It would be difficult to explain the change in alkylglycerol composition on the sole basis of alkylglycerol monoxygenase deficiency, unless the loss involved enzymes that preferentially cleave side chains with C18 groups. Aside from this possibility, which may be tested experimentally, the observed changes would implicate some processes related to ether lipid synthesis. The O-alkylglycerol backbone is formed by substitution of the alcohol for the acyl group of acyldihydroxyacetone phosphate (16). The loss of α-glycerol phosphate dehydrogenase activity in tumors, which might result in greater availability of dihydroxyacetone phosphate (7) and of its acylated derivative, would explain the rise in neutral O-alkylglycerols but it would not explain the observed change in their composition. At least 2 factors may influence the composition of the ether-linked side chains. The first is substrate specificity in the enzymic reduction of fatty acids to fatty alcohols. The more efficient reduction of saturates compared to that of monoenes and of octadecanoic acid compared to that of hexadecanoic acid has been demonstrated in fish roe preparations (12). It is not known whether substrate specificities of this kind are altered in tumors. Secondly, the variable degree to which tumor cells take up preformed fatty acids from their surrounding environment (4, 11, 22) may influence the tissue of the fatty alcohols used for glycerol ether formation. Tumor cells

hepatocellular carcinoma these proportions were altered on the average of 2:1:1. Because both C16 glyceryl ethers were reduced to the same extent, the ratio of alkylglycerols with C16 groups to those with C18 groups was increased almost 6-fold. The proportions of saturated to monoenic alkylglycerols were more than doubled. The extreme values obtained in individual tumor specimens and their corresponding residual tissues showed many of the same changes. The tumors contained higher proportions of hexadecylglycerol and lower proportions of octadecenylglycerol. The C16:C18 glyceryl ethers ratio and the saturates:monoenes ratio were in every case higher in the tumor than in the residual tissue. The lowest C16:C18 ratio found in a hepatocellular carcinoma was 0.43, which was associated with a saturates:monoenes ratio of 2.12; the ratios found in the residual portion of the same specimen were, respectively, 0.14 and 0.95. Statistically significant differences in these ratios could only be demonstrated in the comparison of tumor with Residual Liver A. This was probably due to the smaller number of specimens in the B group.

The abnormalities found in hepatocellular carcinomas were greater in the more necrotic areas. The 2 cases in which a direct comparison was made gave very similar results. Higher concentrations of neutral alkyl glycerolipids and of hexadecaglycerol were present in the lipids from necrotic tissue. In illustration, a specimen of necrotic tumor contained 3.1 µg/g neutral alkyl glycerolipids and 60% hexadecaglycerol; the C16:C18 ratio was 1.81; the saturates:monoenes ratio was 3.17. In the nonnecrotic portion of the same tumor, the concentration of neutral alkylglycerols was 0.5 µg/g, hexadecaglycerol was 45%, the C16:C18 ratio was 0.80, and the saturates:monoenes ratio was 1.84. Since necrosis of single cells or of larger zones is an invariable feature of cancerous tumors (17), we considered the possibility that the observed changes might be associated with the presence of dead tumor cells of any type. This appeared unlikely, because C16:C18 ratios of 0.22 to 0.41 were obtained in preparations from 3 other kinds of tumor specimens, all of which exhibited necrotic changes. These results were: adenosquamous carcinoma, 16:0 (25%), 18:0 (29%), 18:1 (34%), 20:0 (4%), and 22:1 (8%); oat cell carcinoma, 16:0 (26%), 18:0 (47%), and 18:1 (27%); bronchogenic adenocarcinoma, 16:0 (18%), 18:0 (51%), and 18:1 (31%).

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have been found to incorporate extracellular fatty acids into their lipids without much modification of the hydrocarbon chains in the length or degree of saturation (22).

The analyses reported to date of O-alkyl group composition in alkylacylglycerols have been obtained on Ehrlich ascites cells (15), Novikoff hepatoma cells (23), L-M cells (1), Morris hepatomas (17), Harderian gland tumor (8), preputial gland tumors (18), carcinosaoma, taper liver tumor, and lymphosarcoma (13). A C18 glyceryl ether was the predominant component in many of these analyses. These data would suggest that our finding of increased tumor, and lymphosarcoma (13). A C18 glyceryl ether was the predominant component in many of these analyses. These data would suggest that our finding of increased

ACKNOWLEDGMENTS

We are grateful to Professor G. B. Ong for making the surgical specimens of tumor available for this study.

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