Absence of Liver Antigens in Transplantable Hepatomas

A Possible Relationship to the Tumor Aggressiveness

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ABSTRACT

Presence of liver antigens in transplantable Morris hepatomas of varying aggressiveness was investigated by immunodiffusion and absorption methods. The most malignant and undifferentiated of these, hepatoma 7777, was found defective in two liver antigens, whereas the less malignant hepatomas, 5123te and 9618A, did have them. Both antigens had distinct occurrence in tissues and hepatic subcellular fractions and behaved like proteins of electrophoretic mobilities of serum gamma globulins with molecular weights of 51,000 and 240,000, respectively. Since hepatoma 7777 is the least differentiated, fastest growing, and more metastatic of the tumors studied, these properties would appear to be associated with the absence of the antigens.

Introduction

There is growing evidence in animal and human malignancies that the loss of certain antigens in tumors correlates with aggressive biologic behavior and invasiveness.6,16,21,24,27,30,31,34 Early observations on methylcholanthrene-induced murine squamous cell carcinomas indicated that epidermal antigen deletions were more pronounced in deeper parts of the tumors and, at the invading edge, there was almost total depletion of normal epidermal antigens. This suggested that antigen deletion may be a factor in local invasion by malignant cells.27 More recently, loss of cell surface display of major histocompatibility complex has been correlated with the increased metastatic potential of a B16 melanoma variant in mice6 and with the capacity of an SV40 transformed murine fibrosarcoma to grow in immunocompetent as well as in immunosuppressed syngeneic recipients.16 Furthermore, the occasional escape of mouse mastocytoma P815 from host rejection was shown to be related to the development of antigen-loss secondary variants.24 In addition, murine lymphomas that were predominantly H-2D$^{-}$ negative were shown to have more aggressive growth characteristics than their H-2D$^{+}$ positive counterparts.31

In human tumors it has long been recognized that the expression of the blood
group antigens, A, B, and O (H), is frequently lost in squamous and transitional cell carcinoma. Recent reports indicate that in transitional cell carcinoma of the bladder the loss of ABH isoantigens correlates with aggressive behavior and invasiveness. In addition, an antigenic determinant (MoAb145 antigen), that is neither A, B, or H but is shared between human red blood cell membrane and bladder epithelium, has been found to modulate in transitional cell carcinoma of the bladder in a manner similar to that of the isoantigens ABH.

The purpose of this investigation was to determine whether or not such a correlation could be also demonstrated in transplanted liver tumors of varying aggressiveness.

Materials and Methods

TUMORS

Tumors carried in Buffalo-strain rats (Morris hepatomas 7777, 5123tc and 9618A) were used in these studies. Previously reported characteristics of these three tumors are summarized in table I. Tumor line 7777 is a poorly differentiated, fast growing hepatocellular carcinoma induced by dietary administration of N-2-fluorenylphthalamic acid. This line was studied in the 159th transplant generation. Line 5123tc is a tissue culture variant of a moderately differentiated trabecular hepatocellular carcinoma induced with dietary administration of N-2-fluorenyl-phthalamic acid. This tumor is of intermediate rate of growth and was studied in the 165th generation. Tumor line 9618A is a well-differentiated hepatocellular carcinoma induced by 2-(4'-methyl) benzoylaminofluorene. This tumor, of slow rate of growth, was studied in the 13th generation.

Tumor and Normal Rat Organ Extracts

Tumors and normal rat tissues were obtained within two to six minutes after sacrifice. The hepatomas were studied as the tumors reached three cm. The tissues were dissected free of connective tissue and large blood vessels and stored frozen at -60°C. After thawing and mincing two minutes with scissors, they were homogenized in five volumes 0.01 M phosphate buffered NaCl solution at pH 7.2 (PBS) in a Waring Blendor for five minutes at 4°C. After centrifugation at 12,000 rpm for 30 minutes, the supernatant fluid was dialyzed against phosphate buffered saline (PBS) (diluted 1:10 in distilled water) and lyophilized.

Anti-Rat-Liver Serum

The rat liver extract used for immunization was dissolved in PBS in a concentration of 10 mg per ml and emulsified with an equal volume of Freund's complete adjuvant. Albino rabbits were given weekly injections of the emulsion following a schedule previously detailed, and the harvested anti-sera stored in the frozen state. Reaction of these antisera with normal rat serum

Table I

<table>
<thead>
<tr>
<th>Tumor Line</th>
<th>Time (Days) Until Tumor Reached 3 cm</th>
<th>Histology* (Growth Rate Designation)</th>
<th>Metastatic Potential†</th>
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<tbody>
<tr>
<td>7777</td>
<td>18 Poorly differentiated</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>5123tc</td>
<td>35 Moderately differentiated</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>9618A</td>
<td>99-99 Well differentiated</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

constituents was eliminated by absorption with 20 mg of lyophilized, pooled normal rat plasma per ml of antiserum. Such absorbed antisera gave up to seven arcs of precipitation in immunoelectrophoresis with extract of liver. Plasma-absorbed antiliver serum (PAL) giving six strong arcs of precipitation was selected and used routinely in this work. Absorptions followed a procedure indicated earlier.11

**IMMUNODIFFUSION**

Immunoelectrophoresis was performed according to Scheidegger32 using 0.8 percent agarose in 0.025 M veronal buffer at pH 8.2. The electrophoresis was carried out with ice water cooling at 7 V per cm for 60 minutes. Double immunodiffusion was carried out in 0.8 percent agarose gel prepared in PBS containing 0.1 percent sodium azide. Both the double immunodiffusion and immunoelectrophoresis plates were incubated for 24 to 48 hours and photographed.

**LIVER FRACTIONATION PROCEDURES**

Separation of liver cell components into cytosol, microsomes, mitochondria, and nucleus was carried out according to Schneider and Hogeboon.33 Gel filtration of liver extract (60 mg in one ml of PBS) in Sephadex G-200* and Bio-gel A-5m† was performed in 85 × 1.5 cm columns using as eluant PBS containing 0.02 percent sodium azide. Filtration was carried out at 4°C with a flow rate of 10 ml per hour, and fractions of one ml were collected. Blue dextran 2000* was used to define the void volume. Molecular weight was estimated from gel filtration elution volumes according to Porath29 and Laurent and Killander.23

Chromatography on diethylaminoethyl (DEAE) cellulose was performed in a 50 by two cm column using successive stepwise elution with 0.01 M Na₂HPO₄ at pH 7.6 and 0.04 M Na₂HPO₄ at pH 6.8.

Enzymatic treatment of the liver extract with Pronase and trypsin was carried out as previously described.12

**Results**

**ABSENCE OF TWO LIVER ANTIGENS IN FAST-GROWING HEPATOMA 7777**

The plasma-absorbed antiliver serum (PAL) gave five to six lines of precipitation with rat liver extract in double immunodiffusion tests (figure 1, A). Following absorption of PAL with extracts of rat liver, intermediate (5123tc) or slow (9618A) growing hepatomas, no precipitin lines were obtained indicating that all these extracts share the antigens giving the precipitin lines. In figure 1, B are illustrated results obtained with intermediate-growing hepatoma 5123tc. However, absorption with extract of fast-growing hepatoma 7777 failed in the four cases studied to eliminate two of the lines, indicating lack of their corresponding antigens in the tumor (figure 1, C). The minimal concentration of extracts of liver and intermediate and slow-growing hepatoma required for complete inhibition of the lines was 0.5 to 1.0 mg per ml PAL. In contrast, fast-growing hepatoma extracts were ineffective even at a concentration of 100 mg per ml, the maximum dose tested.

The liver antigens found missing in the fast-growing hepatoma were also demonstrated by immunoelectrophoresis where they showed mobilities similar to those of serum gamma-globulins (figure 2). One of these antigens (gamma-1) showed slightly slower mobility than the other (gamma-2).
Properties of Liver Antigens Absent in Hepatoma 7777

Examination of normal rat tissues revealed that antigen gamma-1 is present in kidney and spleen in addition to liver, whereas gamma-2 is liver-specific (table II). Gamma-2 line was inhibited by absorption with as low as 0.5 mg liver extract per ml PAL, whereas extrahepatic tissues were ineffective even at 100 mg per ml.

Antigens gamma-1 and gamma-2 were not detected in livers from dog, cat and mice suggesting rat species specificity.

Studies of the subcellular distribution of the antigens indicated that gamma-1 is localized mainly in cytosol and mitochondrial fractions whereas gamma-2 is more concentrated in mitochondria and equally concentrated in the other fractions (table III).

Liver extracts were treated with Pronase or trypsin. Both antigens were completely inactivated by either enzyme.

Incubation of liver extract for one hour at 4°C in 0.1 M citrate buffer at pH 2.5 resulted in inactivation of both antigens, whereas incubation in phosphate buffers at pH 5.0, 7.0, and 9.0 did not affect them.

Both antigens were shown to be relatively thermolabile. Incubation of liver extract for 30 minutes in PBS at 25°C...
resulted in partial inactivation; treatment at 70°C or higher completely inactivated them.

In Sephadex G-200, gel filtration gamma-1 eluted in fractions corresponding to globular proteins of 52,000 molecular weight (figure 3). The molecular weight of gamma-2 was estimated in a column of Bio-gel A-5m and found to be 240,000 (figure 4).

On DEAE-cellulose chromatography of liver extract, gamma-1 separated from gamma-2, – the former being eluted with 0.01 M Na₂HPO₄ buffer at pH 7.6 whereas the latter eluted with 0.04 M Na₂HPO₄ buffer at pH 6.8.

**TABLE III**

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<tr>
<th>Subcellular Fraction</th>
<th>Antigen Titer*</th>
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<tbody>
<tr>
<td></td>
<td>Gamma-1</td>
</tr>
<tr>
<td>Nuclear</td>
<td>16</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>250</td>
</tr>
<tr>
<td>Microsomes</td>
<td>4</td>
</tr>
<tr>
<td>Cytosol</td>
<td>125</td>
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*Expressed as end point titer in two-fold immuno-diffusion dilution test (d1/g), reciprocal of lowest concentration giving a positive test.

Discussion

This study was undertaken to determine whether or not the correlation between aggressive biologic behavior and loss of antigens found in other tumor models⁶,¹⁶,²⁴,²⁷,³¹ is also demonstrable in transplantable rat hepatomas. Morris hepatomas 7777, 5123tc, and 9618A were selected because these are well characterized tumors exhibiting different growth rates and metastatic properties (see table I).

It was found that two distinct antigenic components of normal rat liver tissue were missing in hepatoma 7777, the most undifferentiated and aggressive of the hepatomas studied. Hepatomas 5123tc and 9618A, which are of more differentiated and of lower aggressive biologic behaviour, showed no qualitative antigenic differences with liver by the immunoelectrophoretic or absorption tests employed. The immunologic methods employed provide only a minimum estimate of the antigenic differences and, therefore, additional antigenic losses may be expected.

It has long been recognized that deletion of liver antigens occurs in carcinogen-induced hepatomas, such as 4-di-
methylaminoazobenzene induced hepatomas. Also such deletions have been shown in hepatomas induced with diethylnitrosamine and 2-acetoamido-fluorene in the rat, and with o-aminozotoluene in the mouse.

Similar observations have been reported with other experimental tumor systems. For example, immunohistochemical studies have shown the loss of kidney antigens in stilbestrol and x-ray-induced kidney tumors, of skin antigens in 3-methylcholanthrene-induced mouse squamous cell carcinoma, and of certain muscle antigens in 20-methylcholanthrene-induced rat rhabdomyosarcoma.

Certain of the deleted antigens in hepatomas have been defined as liver-specific, localized in normal liver microsomes, liver plasma membrane, and soluble cytoplasmic proteins; however, further characterization of these antigens was not reported.

The antigens deleted in the fast-growing hepatoma were characterized as relatively thermolabile and acid pH labile proteins showing distinct molecular weights and tissue and liver cell distribution. Of particular interest was antigen gamma-2 because of its liver specific properties. This antigen behaved in immunoelectrophoresis like a serum gamma-globulin and in Bio-gel A-5m filtration like a protein of 240,000 molecular weight. These properties distinguished gamma-2 from liver specific thermostable antigen, a low density lipoprotein of over 1,000,000 molecular weight, a liver protein of 80,000 molecular weight, and F-antigen of 70,000 molecular weight.

The antigenic changes described here may simply reflect the pronounced modifications of various liver enzymes which are known to occur during carcinogenesis. Weinhouse showed that there is replacement of several liver-type isozymes by non-hepatic types in rapidly growing hepatomas and also noted the loss of such hepatic “marker” isozymes as glucokinase, aldolase, B, pyruvate kinase type L, and adenylate kinase III.

The significance of the antigenic deletions in the malignant behaviour of these and other tumors is an open question since several properties may be considered as contributing factors in tumor aggressiveness. These include the generation times for the tumor cells and the capacity of the tumor cells to induce and/or serve as targets from immune factors. In regard to the former, it can be postulated that the antigenic deletions may reflect an undifferentiated state associated with an increased cellular proliferation rate. With respect to the latter, it is conceivable that one mechanism for the tumors to become more aggressive would be the loss of antigens involved in their immune rejection.

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References

LIVER ANTIGENS IN TRANSPLANTABLE HEPATOMAS


