Abnormal Prothrombin in the Plasma of Rats Carrying Hepatic Tumors

By D.V. Shah, J.A. Engelke, and J.W. Suttie

Vitamin K is required for the synthesis of clotting factors II (prothrombin), VII, IX, and X, and plasma proteins S, C, and Z. The vitamin functions as a cofactor in the posttranslational carboxylation of specific glutamyl residues in microsomal protein precursors to form γ-carboxyglutamyl (Gla) residues in biologically active completed proteins. In the absence of vitamin K or when its action is antagonized by coumarin anticoagulants, des-γ-carboxy or “abnormal” forms of prothrombin are excreted into human and bovine plasma. These proteins differ from prothrombin in their failure to adsorb to insoluble barium salts, to bind calcium ions in solution, or to demonstrate the Ca²⁺-dependent phospholipid association needed for normal physiological activation. Abnormal prothrombin will, however, yield thrombin when treated with a nonphysiological activator such as the protease from Echis carinatus venom.

A survey of the existence of abnormal prothrombin in the plasma of anticoagulant-treated species other than the human or bovine reported substantial amounts in chick, small amounts in rat and mouse, and insignificant amounts in hamster, guinea pig, rabbit, and dog plasma. The basis for the wide species variation in the excretion of abnormal prothrombin has not been determined. Development of a more sensitive amidolytic assay for rat prothrombin has shown that plasma from vitamin K-deficient or warfarin-treated rats contains a non-BaSO₄ adsorbable (undercarboxylated) pool of prothrombin that is equivalent to 5% and 10% of the normal plasma prothrombin concentration. Conformation-specific antibodies directed toward this class of human prothrombin have been developed and used to demonstrate that the concentration of abnormal prothrombin is very low in normal adults, increases substantially with oral anticoagulation or vitamin K deficiency, and is slightly increased in many patients with liver disease.

More recently, it was reported that abnormal prothrombin was more drastically increased in patients with primary hepatocellular carcinoma than in other hepatic disorders and that this response was not due to vitamin K deficiency. The molecular basis for increased levels of abnormal prothrombin in diagnosed cases of hepatic carcinoma has not been established. The response has now been studied using the tumor-bearing rat as a model.

MATERIALS AND METHODS

Treatment of animals. Male rats of the Buffalo strain, weighing 50 to 60 g, were maintained on Purina laboratory chow. Vitamin K-supplemented rats were administered 1 mg of phyloquinone (Konakion, Hoffman-La Roche, Nutley, NJ) intramuscularly (IM) each week for 3 consecutive weeks. Morris hepatoma tumors no. 7777, 5123D, or 9618A were transplanted by disrupting ~1 g of tumor tissue in 5 mL of sterile Dulbecco’s modified Eagle’s medium (DMEM) and giving a 0.5 mL injection in each hind leg of a recipient rat.

Prothrombin and abnormal prothrombin assay. Rat blood for these amidolytic assays was collected by cardiac puncture in 0.15 mol/L of potassium oxalate (10% vol/vol), centrifuged for 20 minutes at 2,000 g at 4 °C, and individual plasma samples were stored at -30 °C. Vitamin K-dependent clotting factors were adsorbed from individual plasma samples by adding 35 mg/mL of citrate-washed BaSO₄ and stirring for 1 hour at 0 °C. Prothrombin and abnormal prothrombin concentrations were determined by assay of thrombin-catalyzed amidolysis of the chromogenic peptide substrate, H-b-Phe-Pip-Arg-pna (S2238 from Kabi Diagnostics, Stockholm) as previously described. Plasma prothrombin (normal) was measured with the chromogenic substrate after activation with a calf serum factor mix and commercial thromboplastin (Thromboplastin, Sigma Diagnostics, St Louis), and total normal plus abnormal prothrombin was measured after activation with E carinatus venom. Abnormal prothrombin was measured by activation of the BaSO₄-adsorbed plasma with E carinatus venom (Sigma).

Vitamin K-dependent carboxylation. Buffalo strain rats carrying the transplantable Morris hepatoma tumors were used when they weighed 160 to 175 g. When administered, sodium warfarin (5 mg/kg) was given by intraperitoneal (IP) injection 18 hours prior to killing. Holtzman strain rats were used as normal controls in carboxylase assay so that the enzyme activities from different rat strains could be standardized. Rats were killed, and a crude microsomal preparation of both liver and tumor tissue was prepared as previously described, except that rats were not fasted. A Triton
X-100 (RPI, Elk Grove Village, IL) solubilized preparation of microsomes equivalent to 0.5 g of liver or 1.0 g of excised tumor mass per milliliter were obtained and incubated at 17 °C for 30 minutes to determine the vitamin K-dependent incorporation of H4CO3 into the substrate Boc-Glu-Glu-Leu-OMe (BaChem Fine Chemicals, Torrance, CA).15

RESULTS

Absorption with BaSO4 is capable of removing all but a small fraction of prothrombin in the plasma of vitamin K-sufficient rats.9 When non-BaSO4 adsorbable prothrombin (abnormal prothrombin) was measured in the plasma of Buffalo strain rats carrying three different Morris hepatoma tumors, a significant increase was seen in the rats carrying the most rapidly growing tumor (Table 1). Buffalo rats carrying the transplantable Morris hepatoma tumor no. 7777 had an approximate fourfold increase in circulating abnormal prothrombin as compared with normal Buffalo rats. Partially carboxylated forms of prothrombin are present following anticoagulation,17,18 and these forms differ in their ability to adsorb to barium salts.19 BaSO4 treatment removed 97% of the thromboplastin-activatable prothrombin in normal plasma but only 85% of this prothrombin species in the plasma of tumor-bearing Buffalo strain rats. These data suggest that a significant amount of partially carboxylated prothrombin is also secreted in tumor-bearing Buffalo strain rats. The concentration of plasma abnormal prothrombin was very much dependent on the period of time after tumor transfer. As shown in Fig 1, normal Buffalo strain rats had 8 to 9 μg/mL of circulating plasma abnormal prothrombin, whereas Buffalo strain rats carrying the transplantable Morris hepatoma tumor no. 7777 had 10 μg/mL at 2 weeks and 33 μg/mL at 3 weeks after transplant. High concentrations of plasma abnormal prothrombin were noted only near the end of the transplant period and presumably reflected tumor mass.

In an attempt to discover the cause of the abnormal prothrombin secretion in tumor-bearing rats, liver and tumor tissue vitamin K-dependent carboxylase activities were determined. Carboxylase activity was slightly higher in the livers of Buffalo strain rats carrying the transplantable Morris hepatoma tumor no. 7777 as compared with normal Buffalo strain rats (Fig 2), but the activity of this enzyme in tumor tissue was <5% of that in normal liver tissue. Liver vitamin K-dependent carboxylase activity is increased by a vitamin K deficiency,14 and carboxylase activity in both normal and tumor-bearing rats and in tumor tissue was depressed when the rats were supplemented with vitamin K (Fig 2). Vitamin K-supplemented Buffalo rats carrying the transplantable Morris hepatoma tumor no. 7777 had the same fourfold increase in circulating abnormal prothrombin as compared with K-supplemented normal Buffalo rats (data not shown) as is shown in Table 1, indicating that the elevated levels of abnormal prothrombin in circulation were not due to vitamin K deficiency.

The response of normal rats to vitamin K deficiency or warfarin administration is an increase in the activity of the liver microsomal vitamin K-dependent carboxylase14 and an increase in the concentration of microsomal prothrombin precursors.20 The livers of rats carrying the no. 7777 tumor demonstrated the same responses, but warfarin administration had no influence on vitamin K-dependent carboxylase activity or microsomal prothrombin precursor concentration of the tumor tissue (Figs 3 and 4).

Rats carrying transplantable Morris hepatoma tumors no.

<table>
<thead>
<tr>
<th>Tumor No.</th>
<th>Weeks Between Transfer</th>
<th>Plasma Prothrombin Concentrations (μg/mL)</th>
<th>Echis carinatus Activation</th>
<th>Thromboplastin Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before BaSO4</td>
<td>After BaSO4</td>
<td>Before BaSO4</td>
</tr>
<tr>
<td>No tumor (12)</td>
<td>—</td>
<td>103 ± 3</td>
<td>9 ± 1</td>
<td>98 ± 2</td>
</tr>
<tr>
<td>7777 (9)</td>
<td>3–4</td>
<td>113 ± 2</td>
<td>34 ± 4‡</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>5123D (12)</td>
<td>6–8</td>
<td>110 ± 3</td>
<td>12 ± 1</td>
<td>112 ± 3</td>
</tr>
<tr>
<td>9618A (9)</td>
<td>8–9</td>
<td>119 ± 3</td>
<td>11 ± 1</td>
<td>110 ± 4</td>
</tr>
</tbody>
</table>

Table 1. Plasma Normal and Abnormal Prothrombin Concentrations in Tumor-Carrying Buffalo Rats

Plasma for analysis was obtained at the time of tumor transfer. The three different tumor strains have different growth rates, and were transferred when the mass of the tumors was similar. Values are mean ± SEM for the number of animals indicated in parentheses.

* "Abnormal" prothrombin.
† "Normal" prothrombin.
‡ Significantly higher (P < .01) than the other three treatments.
Vitamin K-dependent carboxylase activity in liver and tumor tissue of rats. Normal and tumor-bearing Buffalo strain rats were killed 3 weeks after transplant of Morris hepatoma tumor no. 7777. The rats were fed a commercial laboratory chow diet and administered either 1 mg of phylloquinone or saline each week. Vitamin K-dependent carboxylase activity was determined as described in the Materials and Methods section. Data were obtained on different days, and the carboxylase activity of two vitamin K-sufficient Holtzman strain rats assayed each day was used as the 100% control activity. Values are mean ± SEM for 6 to 18 rats in each group. (A) Chow diet only; (B) vitamin K supplement.

5123D and 9618A did not secrete significant amounts of abnormal prothrombin (Table 1). Vitamin K-dependent carboxylase activity and microsomal prothrombin precursor concentration in the livers of normal Buffalo strain rats and the livers of these rats carrying transplanted Morris hepatoma tumors no. 5123D and 9618A were compared. Similar liver carboxylase activities and microsomal precursor concentrations were found (Fig 5) in rats carrying these two tumors. Activity of the vitamin K-dependent carboxylase in the tumor tissue from these rats was, however, ~20% to 30% of that in the host rat liver (Fig 5). This activity was ~15 times higher than the activity observed in the no. 7777 tumor tissue shown in Fig 2. The microsomal prothrombin precursor concentration in the liver of the rats carrying the three
different Morris hepatoma tumors was, however, comparable (Figs 4 and 5), and liver vitamin K-dependent carboxylase activity was somewhat higher in rats carrying the no. 7777 tumor as compared with the 5123D or 9618A tumor (compare Figs 3 and 5).

**DISCUSSION**

Liebman and colleagues detected elevated abnormal prothrombin in the serum of patients with biopsy-confirmed hepatocellular carcinoma and established that this elevation does not reflect a vitamin K deficiency in these patients. The data of the present study demonstrate that the rat is a suitable model for studying this response. Adsorption of oxalated plasma with BaSO₄ will remove almost all normal prothrombin, and increases in venom-generated thrombin above the background represent secretion of abnormal prothrombin. The plasma of rats carrying one of three strains of Morris hepatoma tumors contained significant amounts of abnormal prothrombin.

These data are consistent with the hypothesis that the tumors that increase circulating abnormal prothrombin are those that are capable of expressing the prothrombin gene but that have lost the ability to express significant levels of the vitamin K-dependent carboxylase enzyme. This view is supported by the low concentrations of this enzyme measured in the no. 7777 tumor tissue as compared with much higher concentrations in the 5123D and 9618A lines, which secreted less abnormal prothrombin. It is also possible that the no. 7777 tumor line contains an inhibitor of the enzyme or that turnover rate of the enzyme has been substantially increased. The failure of the no. 7777 tissue to increase carboxylase activity or microsomal prothrombin precursor concentrations following warfarin administration, however, also suggests a low level of expression of this gene. Normal rat liver responds to warfarin administration with an increase in both precursor concentration and enzyme activity, and is evidence that association of the precursor with the enzyme is responsible for these changes. The delay in abnormal prothrombin appearance following transfer of the Morris hepatoma tumors is consistent with the need for a significant tumor mass to develop before this product is seen in plasma.

Vitamin K-dependent carboxylase activity was consistently elevated in the liver of rats carrying tumors. This may reflect the response of carboxylation activity to des-γ-carboxy forms of vitamin K-dependent clotting factors or other unknown factors present in plasma of hypoprothrombinemic animals. Availability of rat hepatoma lines that do secrete large amounts of abnormal prothrombin in the absence of carboxylation provides a valuable tool with which to study this response.

**ACKNOWLEDGMENT**

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