Positive Correlation between Calmodulin Content and Hepatoma Growth Rates

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ABSTRACT

Calmodulin contents of normal rat liver, host liver [bearing hepatoma 5123t.c.(h)], regenerating liver, and Morris hepatomas 7800, 5123t.c.(h), and 7794A were determined by phosphodiesterase assay and by radioimmunoassay. The calmodulin levels determined by both assays were significantly increased in three hepatomas when compared to the corresponding values of normal liver. The order of increase in calmodulin content was as follows: normal liver = host liver < 7794A (slow growth rate) < 5123t.c.(h) (intermediate growth rate) < 7800 (fast growth rate). In regenerating liver (24 hr after partial hepatectomy), the calmodulin content was not different from that of normal liver. In good agreement with the literature, the calmodulin values measured by the phosphodiesterase assay were always lower than those determined by radioimmunoassay. Calcium and magnesium contents were measured by atomic absorption spectrophotometry in acid digests of these tissues. Both cation contents were significantly increased in the three hepatomas studied when compared to the corresponding values of normal liver; the extent of increase for calcium content (120 to 240%) was much greater than that for magnesium (30 to 40%). The order of increase for both cations was as follows: normal liver = host liver < 5123t.c.(h) < 7794A < 7800. Therefore, there does not appear to be any correlation between the cation contents and hepatoma growth rates. In regenerating liver, magnesium content was about 14% higher than that of normal liver, whereas calcium content was not significantly different from normal liver. In summary, the results indicate that only the increase of calmodulin appears to correlate positively with the growth rate of these tumors. This correlation suggests that calmodulin may be involved in tumor cell growth regulation.

INTRODUCTION

The work of Balk et al. (2) strongly suggests that both calcium and magnesium play significant roles in the initiation of cell replication. In neoplastic cells, these workers attribute the lack of cell growth regulation to a defect in the homeostasis of these divalent cations or development of a cation-independent initiation mechanism. Other evidence indicates that extracellular calcium is necessary for DNA synthesis and cell proliferation in normal cells but not in cancer cells (43). At present, the reason(s) for the uncontrolled cell proliferation in tumors is not understood; however, the following possibilities have been suggested (42): (a) cancer cells have abnormally large amounts of cytosolic ionic calcium which can be obtained from subcellular compartments; (b) cancer cells have increased amounts of calcium binding proteins (e.g., calmodulin), through which the initiation of DNA synthesis can be permanently activated without the extracellular calcium surge serving as a trigger.

Additional supporting evidence is provided by Hickie and Kalant (14), who have shown that Morris hepatoma 5123t.c. cells accumulate significant amounts of calcium intracellularly, i.e., more than double the normal concentrations. This finding was considered to be due to increased cell permeability to calcium and binding to intracellular components. Subsequently, other investigators reported increased calcium levels in other tumor cells (1, 34, 38). Recently, Wei and Hickie (40) have reported an increased calmodulin content in supernatant and particulate fractions of Morris hepatoma 5123t.c.(h).

In this paper, we examine the possible correlation between the growth rate of malignant hepatocytes and the levels of calmodulin, calcium, and magnesium. Three Morris hepatomas with varying growth rates have been used in this study, including 7794A (slow growing), 5123t.c.(h) (intermediate growing), and 7800 (fast growing). It is known that the hyperplasia of regenerating liver shares some biochemical characteristics with cancer cells, although regenerating liver still requires extracellular calcium for DNA synthesis (26). Therefore, we also measured calmodulin, calcium, and magnesium levels in regenerating rat liver 24 hr after hepatectomy. This time period was chosen because maximum hepatocyte proliferation occurs at this time (4, 37, 43).

MATERIALS AND METHODS

Reagents. The following reagents were obtained from Sigma Chemical Co., St. Louis, Mo.: 5'-nucleotidase (from Crotalus atrox venom); cAMP1, Tris, calmodulin-deficient CAMP phosphodiesterase (from bovine heart); phosphodiesterase 3',5'-cyclic nucleotide activator (calmodulin, from bovine heart); imidazole; and EGTA. The calmodulin RIA kit was purchased from Caabco, Inc., Houston, Texas. The liver calmodulin, purified from rat according to the procedure of Sharma and Wang (35), was used to construct a calmodulin standard curve. All other chemicals were of highest purity available commercially.

Animals. All animals used in this study were male Buffalorats (200 to 400 g), obtained from Simonsen Laboratories, Gilroy, Calif.

Tumors. The tumors were induced initially in Buffalo rats by feeding various polycyclic amines (31). Hepatoma 5123t.c.(h) was derived from the 5123t.c. subline (15) and was inoculated s.c. and bilaterally in the inguinal region. Tumors 7800 and 7794A were inoculated into trocar (31) into hind leg muscles. These tumors were selected because they have stable growth rates and because there is an extensive fund of information available in relation to their biochemical (29, 30), histological (17), and growth kinetic (20, 21) characteristics. The time

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period between tumor inoculation and use was such that each tumor was permitted to reach a constant size of 5 cm, as measured by the method of Morris and Wagner (31). This time period corresponds to 3 weeks for 7800, 6 weeks for 5123t.c.(h), and 12 weeks for 7794A and appears to be well within the upper limits of the exponential growth curve.

**Regenerating Livers.** Rats were partially hepatectomized by removing the large median and the left lateral liver lobes under ether anesthesia, as described by Higgins and Anderson (16). The removed parts corresponded to 65 to 75% of the liver weight. After 24 hr, the remaining livers were removed for analysis of diveral cations and calmodulin.

**Tissue Calcium and Magnesium Determination.** For the determination of calcium and magnesium contents in liver and hepatoma parenchyma, the tissues were dried in an oven at 90° for 15 hr. Known weights of dried tissue were digested in concentrated nitric acid (containing 0.5% lanthanum chloride) by heating at 70° in an oven for 1 hr. The acid digests were assayed for calcium and magnesium contents in a Perkin-Elmer Model 272 or 500 atomic absorption spectrophotometer using a air/acetylene flame.

**Preparation of Tissue Extracts for Calmodulin Assay.** Rat livers and hepatomas were collected on ice and kept in a homogenizing buffer (which contained 125 mM borate, 1 mM EGTA, and 75 mM NaCl, pH 8.4). The tissues were suspended in this buffer (1 part tissue:5 parts buffer) and homogenized with a motor-driven glass-Teflon pestle using 7 up-and-down strokes. Following homogenization, appropriate aliquots were removed for total protein determination. The remaining homogenates were heat treated at 90° for 5 min and quickly cooled on ice. The heat-treated samples were then centrifuged at 100,000 x g for 30 min, and the supernatant solutions were used for calmodulin assay.

The percentage of total calmodulin extracted by this procedure, including homogenization and heat treatment, was approximately 97% for the tissues studied. This finding is in agreement with that of Dr. J. E. Chafouleas,4 who has found that calmodulin recovery from a variety of tissues using this method is 98 to 100%. If EGTA is absent from the homogenizing buffer, only about 60% of the total tissue calmodulin will be extracted.

**PDE Assay for Calmodulin.** The tissue calmodulin content was assayed according to the method of Sharma and Wang (35) using calmodulin-deficient cAMP PDE, in which the stimulation of this enzyme (+Ca2+) was compared to a standard curve constructed with purified calmodulin. The samples from tissue extracts were assayed in duplicate. The PDE activity was measured at 30° (pH 7.5) in a reaction mixture containing 5 mM borate, 3 mM NaCl, 40 mM Tris, 40 mM imidazole, 5 mM magnesium acetate, 1.2 mM cAMP, 0.3 units of 5'-nucleotidase, 0.016 units of calmodulin-deficient PDE, and either 0.1 mM Ca2+ or 0.1 mM EGTA. The inorganic phosphate released in the reaction was measured by the method of Finke and Subbarow (11).

**RIA for Calmodulin.** The tissue calmodulin content was assayed according to the method of Chafouleas et al. (6). The samples from tissue extracts were assayed in duplicate. Appropriately diluted samples (100 µl) were mixed with a fixed concentration of 125I-calmodulin (10,000 cpm) in an RIA buffer [125 mM borate:bovine serum albumin (20 µg/ml):1 mM EGTA:75 mM NaCl, pH 8.0] and incubated with anti-calmodulin (1.5 µg/assay tube; 40% total bound) in a total volume of 500 µl. Samples were incubated for 18 hr at 25°. Then, 25 µl of a 10% Pansorbin suspension were added. All samples were incubated for another 30 min at 25° followed by centrifugation at 10,000 X g for 15 min. The pellets were washed twice with 1-m1 aliquots of the RIA buffer. Radioactivity of the resulting pellets was determined in a Beckman Biogamma counter. The calmodulin contents of the tissue extracts were compared to a calmodulin standard curve constructed with purified calmodulin.

**RESULTS**

**Wet Weight, Dry Weight, and Protein Content of Livers and Hepatomas.** The water content was found to be higher in tumors than in normal tissues (10, 13, 14, 38). For the appropriate expression of the experimental data in this study, we felt that it was necessary to determine the wet weight, dry weight, and protein content of each of the tissues studied. These data are summarized in Table 1. The dry weight and protein content per g wet weight of host liver and regenerating liver were similar to those of normal liver. However, with the hepatomas, there was a significant decrease in dry weight and protein content. The order of the protein content in these tissues is as follows: normal liver (100%) > 7794A (82.8%) > 5123t.c.(h) (72.5%) > 7800 (66.1%). The decrease in the dry weight and protein content of the hepatomas is probably related to the higher water content in these tissues. Since the water content is increased substantially in the hepatomas, and since it is difficult to determine wet weight accurately, it is concluded that either dry weight or protein content is a more appropriate unit for expressing our data than is wet weight.

**Calcium, Magnesium, and Calmodulin Content of Normal Liver and Regenerating Liver.** Partial hepatectomy is a classical method for inducing cell proliferation in the liver. It has been well documented that, within 24 hr after hepatectomy in rats, the remaining liver lobe(s) doubles in size (5, 16, 36). Peak DNA synthesis takes place between 18 and 24 hr posthepatectomy, and peak mitotic activity of hepatic cells occurs between 24 and 28 hr after hepatectomy (4, 12, 37). The molecular events responsible for this controlled hyperplasia still remain largely unknown. It was of interest to determine whether calcium, magnesium, and calmodulin levels in regenerating liver are altered during the peak mitotic activity. The results of this study (Table 2) indicate that there is no significant change in calcium or calmodulin content in regenerating liver 24 hr after hepatectomy; there is, however, a small, but significant, increase (14%) in magnesium levels.

**Calcium, Magnesium, and Calmodulin Content of Normal Liver, Host Liver, and Hepatomas.** The calcium and magnesium contents of host liver were not significantly different from those of normal liver (Table 3). In contrast, the calcium contents of hepatomas were significantly higher than those of normal or host liver. The order of these increases is as follows: normal liver < 5123t.c.(h) (120%) < 7794A (167%) < 7800 (241%).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Wet wt (g)</th>
<th>Dry wt (mg)</th>
<th>Protein content (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal liver</td>
<td>339 ± 5.0</td>
<td>170.6 ± 4.0</td>
<td></td>
</tr>
<tr>
<td>Host liverc</td>
<td>338 ± 2.0</td>
<td>172.7 ± 6.4</td>
<td></td>
</tr>
<tr>
<td>Regenerating liver</td>
<td>342 ± 9.1</td>
<td>174.5 ± 7.9</td>
<td></td>
</tr>
<tr>
<td>Hepatoma 7800</td>
<td>221 ± 14.3c</td>
<td>112.8 ± 2.8c</td>
<td></td>
</tr>
<tr>
<td>Hepatoma 5123t.c.(h)</td>
<td>227 ± 8.1c</td>
<td>123.0 ± 5.1c</td>
<td></td>
</tr>
<tr>
<td>Hepatoma 7794A</td>
<td>204 ± 10.2c</td>
<td>141.3 ± 7.0c</td>
<td></td>
</tr>
</tbody>
</table>

Table 1

**Protein Determination.** Protein was determined by the method of Lowry et al. (22) using bovine serum albumin as a standard.

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4 J. G. Chafouleas, personal communication.

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The magnesium contents of hepatomas were also significantly higher than those of normal liver; however, the extent of this increase was less than that of calcium (i.e., 30 to 40% increase). It is evident that the increase in calcium and magnesium contents did not correlate with the tumor growth rate.

The calmodulin values measured by the PDE assay were always 2.2- to 2.7-fold higher than those obtained by the PDE assay (Table 4). This finding was consistent with the results of Chafouleas et al. (6). The calmodulin content of hepatomas was significantly higher than that of normal liver (Table 4). The order of this increase, as determined by the PDE assay, is as follows: normal liver < 175.8 µg/g (64%) < 5123t.c.(h) (102%) < 7800 (184%); whereas the order and the percentage of increase as measured by RIA are as follows: normal liver < 0.221 µg/mg (4%) < 0.628 µg/mg (24%) < 0.447 µg/mg (2%) < 0.363 µg/mg (2%). Therefore, it appears that the increase in calmodulin content is positively correlated with the growth rate of these tumors.

**DISCUSSION**

The results of the present study indicate that a positive correlation exists between the growth rate of 3 Morris hepatomas and their corresponding levels of calmodulin. This is true regardless of the method used to measure calmodulin. These findings suggest the involvement of calmodulin in tumor cell growth regulation.

There are several lines of evidence now implicating the involvement of calmodulin in the process of cell mitosis. For example, means and Dedman (28) and Welsh et al. (41) have shown that calmodulin reversibly associates with the mitotic apparatus and is approntly involved in regulating microtubule disassembly. Recent studies by Boynton et al. (3) suggest that calmodulin stimulates DNA synthesis in rat hepatocytes in vitro. The calmodulin levels are also elevated in exponentially growing transformed cells (7, 19, 39). The present results further strengthen our previous findings that the increased calmodulin levels in hepatoma 5123t.c.(h) may contribute to the abnormal proliferation of hepatoma cells (40).

In regenerating liver 24 hr after hepatectomy, the calmodulin levels did not show significant change. Therefore, the prolonged abnormally high level of calmodulin may be associated only with tumors. After completing this work, a report appeared indicating a significant but temporary increase in calmodulin content in regenerating liver 4 to 6 hr after hepatectomy (27); this report also supports our previous finding (40) and indicates that calmodulin levels are increased significantly in other hepatomas as well.

The calmodulin levels detected by RIA were higher than those measured by the PDE assay, which is consistent with the studies of Chafouleas et al. (6). The reason(s) for these differences is not known at present; however, one contributing factor may be the assay conditions. That is, the determination of calmodulin levels by the PDE assay is dependent upon the activation of PDE by calmodulin in a calcium-dependent manner. Intrinsic to this assay is the requirement for biologically active calmodulin and calcium-dependent binding. In the RIA assay, the calmodulin:anticalmodulin interaction is, however, calcium independent. Thus, the different pools of biologically active calmodulin or heat-stable calmodulin binding proteins (that interact with calmodulin in a calcium-dependent manner) may be influenced by the calmodulin activity detected by the PDE assay but not by RIA.

It is evident that no direct correlation exists between the growth rates of the 3 Morris hepatomas studied and their corresponding levels of calcium and magnesium. However, these levels are higher than those of normal liver for all 3 hepatomas studied, particularly for the rapidly growing tumor 7800. This lack of correlation does not negate the possible involvement of the divalent cations in cell growth regulation since other changes may have taken place in tumors, which could modify the effect of divalent cations, for example, changes in calcium binding proteins (24, 25, 27, 40), prostaglandin levels (18), and cyclic nucleotide-dependent protein kinases (8, 9, 23). Furthermore, differences in total cellular magnesium and calcium may not reflect possible differences that exist in the ionized concentrations of these elements in the cytosol.

**Table 2**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Calcium content (µg/g dry wt)</th>
<th>Magnesium content (µg/g dry wt)</th>
<th>Calmodulin content (µg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal liver</td>
<td>170.0 ± 1.6a</td>
<td>707.0 ± 14.9</td>
<td>0.241 ± 0.011</td>
</tr>
<tr>
<td>Regenerating liver</td>
<td>168.6 ± 1.9</td>
<td>804.0 ± 33.4</td>
<td>0.244 ± 0.010</td>
</tr>
</tbody>
</table>

*Mean ± S.E.; the number of observations is 6.

*The value was significantly different (p < 0.05) from the corresponding value of normal liver, as measured by Student's t test.

**Table 3**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Calcium content (µg/g dry wt)</th>
<th>Magnesium content (µg/g dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal liver</td>
<td>175.8 ± 4.1b</td>
<td>706.1 ± 21.8</td>
</tr>
<tr>
<td>Host liver</td>
<td>172.0 ± 4.5</td>
<td>737.2 ± 23.0</td>
</tr>
<tr>
<td>Hepatoma 7800</td>
<td>600.0 ± 80.5d</td>
<td>1002.6 ± 95.3c</td>
</tr>
<tr>
<td>Hepatoma 5123t.c.(h)</td>
<td>387.1 ± 27.4b</td>
<td>817.2 ± 63.3p</td>
</tr>
<tr>
<td>Hepatoma 7794A</td>
<td>476.0 ± 41.4b</td>
<td>927.2 ± 94.2p</td>
</tr>
</tbody>
</table>

*Mean ± S.E.; the number of observations is 6.

*The value was significantly different (p < 0.05) from the corresponding value of normal liver, as measured by Student's t test.

**Table 4**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Calmodulin content (µg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal liver</td>
<td>0.221 ± 0.013a</td>
</tr>
<tr>
<td>Host liver</td>
<td>0.238 ± 0.037</td>
</tr>
<tr>
<td>Hepatoma 7800</td>
<td>0.628 ± 0.036b</td>
</tr>
<tr>
<td>Hepatoma 5123t.c.(h)</td>
<td>0.447 ± 0.021b</td>
</tr>
<tr>
<td>Hepatoma 7794A</td>
<td>0.363 ± 0.072b</td>
</tr>
</tbody>
</table>

*Mean ± S.E.; the number of observations is 6.

*The value was significantly different (p < 0.05) from the corresponding value of normal liver, as measured by Student's t test.
In regenerating liver 24 hr after hepatectomy, the calcium level did not change significantly; however, magnesium showed a slight, but significant, increase (14%). It has been reported that a temporary calcium surge into the cells is required for the initiation of DNA synthesis during liver regeneration approximately 12 hr after hepatectomy (43). The increase of magnesium content in regenerating liver 24 hr after hepatectomy probably reflects the rapid synthesis of DNA and/or protein (32). The precise mechanism responsible for this increase is not clear at this time. Rubin (33) has proposed that magnesium may play a key role in regulating metabolism and growth of mammalian cells.

The results presented in Table 1 indicate that there is a significant decrease in dry weight and protein content of hepatomas. These decreases are probably due to increased water content in hepatomas. From the results of Guillino et al. (13), it seems highly probable that the increase in water content of these tumors reflects an enlarged interstitial water compartment. The difference in water content between tumors and livers raises the intriguing possibility that anticalmodulin drugs might inhibit tumor cell growth in vitro or in vivo.

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