Polyamine Depletion of the MTW9 Mammary Tumor and Subsequent Elevation of Spermidine in the Sera of Tumor-bearing Rats as a Biochemical Marker of Tumor Regression

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SUMMARY

Polyamine levels were measured in livers, tumors, sera, and tumor interstitial fluids of rats with growing mammary tumors, as well as in rats at various times after the initiation of tumor regression obtained by hormonal deprivation. Although there were traces of putrescine and spermine in the sera of tumor-bearing animals, spermidine was the only polyamine that could be found consistently in measurable amounts. Decreases in both liver and tumor levels of spermidine parallel the elevation of spermidine in the serum. The period of maximal tumor regression, i.e., within 48 hr of removal of hormonal support, corresponds with the time of the highest levels of spermidine in serum and in tumor interstitial fluid. These data indicate that intracellular spermidine levels, which increase during tumor growth, are lowered by excretion during regression and that spermidine levels in the serum reflect tumor cell death.

INTRODUCTION

There is increasing interest in defining biochemical markers that will allow the clinician to diagnose cancer in its early stages or to evaluate the effects of various chemical or radiation regimens upon tumor cells. Preliminary data from studies of cancer patients suggest that there is a relationship between tumor metabolism and the levels of polyamines in the urine (1, 11, 12, 14, 17). Patients diagnosed as having cancer excrete increased amounts of polyamines. A considerable fraction of the polyamines exists in the urine as conjugates since hydrolysis is necessary in order to liberate the free bases (14). Surgical removal of a portion of the tumor mass decreases the urinary levels of the polyamines; this is consistent with the hypothesis that the urinary polyamines are a product of the tumor (14). In addition, elevated polyamine levels have been detected in the sera of cancer patients (10). This is now possible using very sensitive techniques, such as dansylation followed by thin-layer chromatography or an amino acid analyzer technique (7, 10).

It is possible that polyamine levels in the sera of cancer patients may serve as indicators for evaluating the efficacy of chemotherapy and/or radiation therapy. To test this possibility, we have studied an animal model system, the MTW9 mammary tumor of the rat. This tumor was produced by Kim and Furth (9) and is ideal for study because it is hormone dependent and the course of regression, which is rapid, can be studied after removal of the source of the mammotrophic hormone (5). The time course of the regression of this tumor and changes in the biochemical parameters of the tumor (5) have been studied extensively. Polyamine levels were assessed in the livers, tumors, sera, and the interstitial fluids of the mammary tumors at various times after the removal of a pituitary tumor which sustains the growth of the mammary tumor (5).

MATERIALS AND METHODS

Putrescine, spermidine, and spermine hydrochlorides were obtained from Calbiochem (Los Angeles, Calif.) and recrystallized 3 times from ethanol before use. 14C-Labeled polyamines, which were used to determine recovery rates of the polyamines, were obtained from New England Nuclear, (Boston, Mass.). Buffers for the amino acid analyzer were prepared from Beckman buffer concentrates and the appropriate amounts of sodium chloride were added to adjust the molarity. Ninhydrin solutions were made from prepackaged ninhydrin kits from BioRad Laboratories (Richmond, Calif.). Animals were castrated under ether anesthesia.

Polyamines in plasma, serum, and interstitial fluid were assayed as previously described (10). There were no detectable differences in the concentrations of polyamines present in plasma as compared to serum. Therefore, the data reported are

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for sera. Further, there were no significant differences between arterial and venous samples. Tumor and liver tissues were homogenized in 4 volumes of cold 0.1 N HCl in a Duall tissue grinder. Dry sulfosalicylic acid was added to a final concentration of 4%. The homogenates were centrifuged at 1000 X g for 15 min. An aliquot of a supernatant was assayed for individual polyamines after separation by a Beckman Model 121 automatic amino acid analyzer as previously described (10).

Recovery of the polyamines by this method was 98 to 100%.

**Tumor Transplantation.** MTW9 mammary carcinomas were transplanted into the inguinal fat pad of 170- to 190-g female Sprague-Dawley rats. After the tumor grew to the size of a pea, the tumor and vascular pedicle supplying it with blood were surgically isolated following a procedure previously described (2). A preparation was obtained in which the tumor grew in a paraffin envelope and was connected to the host by only 1 artery and 1 vein. Growth of MTW9 was maintained by high blood levels of mammotrophins. A pituitary tumor (MtTW10) was used as the source of mammotrophins and was transplanted into the interscapular area at the same time MTW9 was transplanted into the inguinal fat pad. Removal of MtTW10 and castration produced regression of MTW9 (5).

Tumors of 3 to 4 g were utilized in this study. Plasma samples (1 to 2 ml) were obtained from heparinized animals, and the blood was centrifuged at 5°; serum (1 to 2 ml/sample) was prepared from nonheparinized animals after 30 min clotting at room temperature.

**Sampling Procedures.** The rat was anesthetized with urethan (1.0 mg/g) and then heparinized (1.0 mg/200 g body weight). One PE50 catheter (Clay-Adams, Parsippany, N. J.) was placed in the tumor vein, a 2nd catheter was placed into the thoracic aorta via the left carotid artery, and a 3rd catheter was placed into the right external jugular vein. Blood draining out of the tumor was collected continuously for 1 hr. Through the catheter located in the jugular vein, blood taken from intact animals was introduced in quantities about equal to that removed through the tumor vein. Thus, constant arterial pressure was maintained during sampling. From the aortic catheter, arterial samples were taken at the beginning and at every 20 min thereafter. At the end of the experiment, the rat was sacrificed, the tumor plus a fragment of the left lobe of the liver were removed, and these tissues were frozen at —20°.

The TIF (4) was sampled following a procedure previously described (3). A pouch about 2 x 2 cm was prepared in the s.c. tissues of the left inguinal area. A 1.5-cm micropore chamber with a catheter attached was inserted into the pouch and surrounded by tumor fragments of about 1 cu mm. As the tumor grew, it incorporated the micropore chamber. Neoplastic cells are not able to penetrate into the chamber which has walls constituted by micropore filters of 0.45-μm pore diameter, but the fluid surrounding the cells is able to pass through the pores and was sampled by means of the catheter for the chamber. The sample is obtained by placing the animal in a restraining cage. The amount collected in a 24-hr period was 0.2 to 1.0 ml. An extensive treatment of the methodology has been published (4).

In 3 groups of 4 animals each, MTW9 tumors incorporating micropore chambers were prepared. One sample of TIF was removed from each tumor during growth; regression was induced and TIF was sampled again. Thus, 2 samples were obtained from the same tumor, 1 during growth and 1 at 24 (Group 1), 48 (Group 2), or 72 (Group 3) hr of regression. After the 2nd TIF sample was taken, the animal was anesthetized, serum of aortic blood was collected, and the left lobe of the liver was removed and frozen at —20°.

**RESULTS**

**Polyamines in Serum.** Spermidine was the only polyamine that could be consistently measured in the serum of rats. Castration alone was followed by a small increment in serum spermidine levels (Chart 1). However, in tumor-bearing rats, the initial spermidine level was higher than that present in normal rats and, after castration and the removal of the MtTW10 pituitary tumor that supports the tumor growth, the serum level of spermidine increased for about 36 hr and then declined. The tumor volume was reduced to one-half in 48 to 72 hr.

**Polyamine Concentrations in MTW9 Tumors, in Corresponding Livers, and in Sera at Various Times after the Initiation of Tumor Regression.** Table 1 compares the concentrations of polyamines in tumors, livers, and sera of rats with growing tumors and at various times after the initiation of tumor regression. The ratio of spermidine to spermine in both the tumor and the liver is highest in animals with growing tumors. Within 24 hr after the initiation of the regression process, the spermidine/spermine ratio for the tumor dropped from 4.2 to 2.7 and for the liver of the tumor-bearing animals from 3.9 to 3.1. The decrease in this ratio appears to be due to a decrease in the spermidine levels in the tumors and the livers, as well as to increases in spermine levels. Within 48 hr after the initiation of tumor regression, tumor speridine decreased to 67% of control. The level of spermidine in the liver of tumor-bearing animals decreased to 73% of controls. At the same time, serum spermidine levels doubled. Within 72 hr of regression, the ratio of spermidine to spermine in the tumor
dropped to 1.8 and spermidine remained elevated in the serum.

In order to determine whether any of the serum spermidine is due to release from the tumor, polyamine concentrations were assayed in the TIF before and after initiation of regression (Table 2). Although there is considerable variation in the levels of spermidine detectable in the sera of rats with growing and regressing tumors, in all cases the concentration of spermidine in the TIF is higher in animals with regressing tumors. Further, a relatively high concentration of spermidine occurs in TIF 48 hr after the initiation of regression, and this again correlates well with the decreased spermidine concentration measured in the tumor, as well as the increased concentration of spermidine detectable in the serum.

Table 1
Putrescine, spermidine, and spermine concentrations in MTW9 mammary tumors, livers, and sera of rats at various times after the initiation of tumor regression

Polyamine concentrations were measured with a Beckman Model 121 automatic amino acid analyzer (Ref. 6; see "Materials and Methods"). The sensitivity of this method is no greater than 1 n mole.

<table>
<thead>
<tr>
<th>Status</th>
<th>Putrescine (nmoles/g wet wt)</th>
<th>Spermidine (nmoles/g wet wt)</th>
<th>Spermine (nmoles/g wet wt)</th>
<th>Spermine (nmoles/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>Growing</td>
<td>77 ± 2.9°</td>
<td>1570 ± 26</td>
<td>372 ± 14</td>
</tr>
<tr>
<td>Liver</td>
<td>26 ± 1.1</td>
<td>2550 ± 84</td>
<td>655 ± 18</td>
<td>3.9</td>
</tr>
<tr>
<td>Serum</td>
<td>NDb</td>
<td>3.8 ± 0.9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Tumor</td>
<td>24-hr regressing</td>
<td>45 ± 0.75</td>
<td>1480 ± 30</td>
<td>546 ± 20</td>
</tr>
<tr>
<td>Liver</td>
<td>&lt;20</td>
<td>2270 ± 73</td>
<td>721 ± 19</td>
<td>2.7</td>
</tr>
<tr>
<td>Serum</td>
<td>1.7 ± 0.40</td>
<td>7.3 ± 1.8</td>
<td>ND</td>
<td>3.1</td>
</tr>
<tr>
<td>Tumor</td>
<td>48-hr regressing</td>
<td>32 ± 3.1</td>
<td>1040 ± 100</td>
<td>483 ± 95</td>
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<tr>
<td>Liver</td>
<td>&lt;20</td>
<td>1870 ± 50</td>
<td>755 ± 29</td>
<td>2.5</td>
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<tr>
<td>Serum</td>
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<td>11.2 ± 2.2</td>
<td>0.18 ± 0.02</td>
<td>ND</td>
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<tr>
<td>Tumor</td>
<td>72-hr regressing</td>
<td>39 ± 2.5</td>
<td>980 ± 44</td>
<td>554 ± 24</td>
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<tr>
<td>Liver</td>
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<td>1590 ± 35</td>
<td>782 ± 43</td>
<td>1.8</td>
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<tr>
<td>Serum</td>
<td>1.9 ± 0.2</td>
<td>7.2 ± 0.8</td>
<td>0.18 ± 0.03</td>
<td>ND</td>
</tr>
</tbody>
</table>

aMean ± S.E. of samples from 4 separate rats.
bND, amine was not detectable.

Table 2
Putrescine, spermidine, and spermine concentrations in MTW9 mammary TIF before and after initiation of regression

The method of obtaining TIF is specified under "Sampling Procedures." Two samples were obtained from the same tumor, 1 during growth and the 1 at 24 (Group 1), 48 (Group 2), or 72 (Group 3) hr of regression. Polyamine levels were measured with a Beckman Model 121 automatic amino acid analyzer (Ref. 6; see "Materials and Methods").

<table>
<thead>
<tr>
<th>Group</th>
<th>Putrescine (nmoles/ml)</th>
<th>Spermidine (nmoles/ml)</th>
<th>Spermine (nmoles/ml)</th>
<th>Time after initiation of regression (hr)</th>
<th>Putrescine (nmoles/ml)</th>
<th>Spermidine (nmoles/ml)</th>
<th>Spermine (nmoles/ml)</th>
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<td>1</td>
<td>2.9</td>
<td>6.6</td>
<td>NDb</td>
<td>24</td>
<td>1.7</td>
<td>7.6</td>
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<tr>
<td>1</td>
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<td>2.0</td>
<td>ND</td>
<td>4.4</td>
<td>8.3</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>1</td>
<td>9.8</td>
<td>9.9</td>
<td>ND</td>
<td>3.9</td>
<td>10.5</td>
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<td>ND</td>
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<tr>
<td>2</td>
<td>7.1 ± 1.9</td>
<td>3.4 ± 0.6</td>
<td>9.4 ± 0.8</td>
<td>22</td>
<td>11.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>3.6</td>
<td>8.7</td>
<td>ND</td>
<td>48</td>
<td>2.2</td>
<td>11.4</td>
<td>ND</td>
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<tr>
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<td>6.3</td>
<td>9.3</td>
<td>ND</td>
<td>4.4</td>
<td>13.3</td>
<td>+</td>
<td>ND</td>
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<tr>
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<td>5.4</td>
<td>5.9</td>
<td>ND</td>
<td>2.9</td>
<td>11.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>6.6 ± 0.7</td>
<td>3.1 ± 0.5</td>
<td>10.8 ± 1.3°</td>
<td>72</td>
<td>1.8</td>
<td>5.9</td>
<td>ND</td>
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<tr>
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<td>3.7</td>
<td>ND</td>
<td>1.8</td>
<td>5.9</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>3</td>
<td>6.7</td>
<td>3.6</td>
<td>ND</td>
<td>2.9</td>
<td>7.6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>7.9</td>
<td>2.6</td>
<td>2.3</td>
<td>7.9</td>
<td>1.1</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

aND, not detectable; +, amine was present but too low to quantitate.
bMean ± S.E.
cData differ from control (growing tumor); p < 0.05.
DISCUSSION

The demonstration of putrescine, spermidine, and sometimes spermine in the interstitial fluid of growing tumors at a level higher than in serum is the first direct evidence that at least a portion of the polyamines in the serum are derived from tumor tissue. As previously stated, spermidine is the only polyamine with a concentration high enough to be measured. However, the method utilized is sensitive only to the 1-n mole range. During tumor regression, elevations of spermidine in both TIF and serum correspond to the period of maximal tumor regression; e.g., within 48 hr after removal of the source of mammotrophic hormone. Within an average of 60 hr after the initiation of tumor regression, the tumor volume is reduced to one-half that at the start.

This area is so new and of such potential interest to oncologists that some speculation is in order. There are changes in the spermidine concentrations of the livers of the tumor-bearing rats after initiation of tumor regression. The early changes (within 24 hr) may involve a conversion of spermidine to spermine (Table 1) as has been previously reported (8). Within 48 hr there is a further decrease in the spermidine level of the liver and this also may be reflected in the elevation of spermidine in serum. An increase in the level of spermidine in the liver of tumor-bearing mice and rats has been demonstrated previously (6, 15). These increases are due to tumor cell invasion in the case of leukemic mice (6), and they could be due to tumor cell invasion in rats with flank-injected hepatomas (15). However, in the latter case, it seems more probable that the spermidine increase in the liver is in response to increased metabolic demand on the liver. Once this demand slackens, in this case during tumor regression, the spermidine level decreases.

High spermidine/spermine ratios are characteristic of metabolically active tissues or tissues that are undergoing rapid proliferation (8, 16). The ratio of spermidine to spermine in normal rat liver is near 1. Both the tumor and liver of rats with mammary tumors have ratios about 4 (Table 1). After 72 hr of regression, the ratio of spermidine to spermine is reduced to 1.8 in the mammary tumor and to 2.0 in the liver. This further emphasizes the intimate connection between tumor and liver metabolism.

Perhaps it is possible to think in terms of the entire serum spermidine concentration as being a function of tumor metabolism, whether the spermidine is derived from tumor tissue or from the liver of tumor-bearing rats. Further studies are necessary in order to ascertain whether total tumor burden and/or fluctuations in total tumor as a result of chemotherapy, radiation therapy, or other regimens resulting in tumor regression can be assessed as a function of serum or urine polyamine levels.

REFERENCES
Polyamine Depletion of the MTW9 Mammary Tumor and Subsequent Elevation of Spermidine in the Sera of Tumor-bearing Rats as a Biochemical Marker of Tumor Regression


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