Changes in Polyamine Levels and Protein Synthesis Rate during Rat Liver Carcinogenesis Induced by 4-Dimethylaminoazobenzene

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ABSTRACT

The concentrations of putrescine, spermidine, and spermine in liver of rats fed on 4-dimethylaminoazobenzene and in the resultant hepatomas were found to be significantly higher than were those observed in normal liver from rats of the same strain, sex, and age. These modifications were due to the carcinogen and not to the special low-riboflavin diet used to obtain the carcinogenic effect of 4-dimethylaminobenzene. The first change observed during liver carcinogenesis was the early increase in the putrescine level, followed by an increase of spermidine and spermine, which reached maximum levels in growing hepatomas. A significant increase of urinary polyamines was also observed in tumor-bearing rats. Experiments on leucine incorporation into proteins of tissue slices, which were obtained from the same tissues on which polyamine determinations were carried out, showed that in rat liver carcinogenesis the rate of protein synthesis was well correlated with the polyamine levels. These results suggest that polyamines may play a role in the process of carcinogenesis and in tumor protein synthesis in vivo.

INTRODUCTION

Several observations (1, 2, 24, 28, 35, 36) indicate that the polyamines (putrescine, spermidine, and spermine) have important functions in cellular metabolism and are intimately associated with many aspects of the growth of normal and neoplastic cells. An increase of polyamine levels and the activation of ornithine decarboxylase (EC 4.1.1.17), the rate-limiting enzyme in the biosynthetic pathway of these substances (14, 31), has been described in rapidly growing tissues such as embryonic tissues, regenerating liver, target organs after appropriate hormone administration, and some tumors (1, 25, 37). Although the exact role of polyamines in cell growth remains to be elucidated, there is evidence that these intracellular polyamines are of importance in the regulation of the synthesis of DNA (8, 18, 32, 34), RNA (4), and proteins (12, 35, 36).

This investigation was designed (a) to provide information about the polyamine levels in a tissue during the process of carcinogenesis and (b) to determine whether in this process there is a positive correlation between the polyamine level and the rate of protein synthesis. The levels of putrescine, spermidine, and spermine were determined at various intervals after 4-DAB treatment in rat livers and in the resultant hepatomas. The possible correlation between polyamine levels and the rate of protein synthesis was examined by comparison of the polyamine concentrations and the rate of labeled leucine incorporation into proteins of slices obtained from the same tissues.

MATERIALS AND METHODS

Chemicals. Unlabeled putrescine dihydrochloride, spermidine trihydrochloride, and spermine tetrahydrochloride were obtained from Fluka, Buchs, Switzerland. 1,4-[14C]-Putrescine dihydrochloride (specific activity, 17.8 mCi/mmol), [14C]spermidine trihydrochloride (specific activity, 122 mCi/mmol), [14C]spermine tetrahydrochloride (specific activity, 112 mCi/mmol), and L-[1-14C]leucine (specific activity, 56 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, England. Unlabeled L-leucine was obtained from Sigma Chemical Co., St. Louis, Mo. Other chemicals were analytical-grade products.

Animals. Male Sprague-Dawley rats, 30 days old and weighing 70 to 80 g, were divided into 3 groups: rats fed on a commercial standard diet (controls), rats fed on a Miller and Miller diet (19) without carcinogen (white Millers' diet), and rats fed on a Miller and Miller diet containing 0.06% 4-DAB (4-DAB diet) (19).

At specified intervals during the diet administration, fed rats were killed by decapitation at 10 a.m. In control rats and in rats fed on white Millers' diet, the experiments were carried out on liver samples. In 4-DAB-fed rats the experiments were carried out on livers that, up to 60 days of the diet, were apparently normal and on livers with small tumor nodules of about 2 to 4 mm in diameter that appeared after 90 days of the 4-DAB diet. After 120, 150, and 180 days of the carcinogenic diet, only small tumors 5 to 8 mm, tumors about 10 to 20 mm, and large tumors greater than 25 mm in diameter, respectively, were used.

For the study of polyamine levels in urine, rats fed on 4-DAB for 150 days and control rats of the same age (6 months) were placed in individual metabolic cages, and 24-hr urines were collected in the cold under toluene (9). Among the animals fed on 4-DAB, only urine samples from rats with hepatomas diagnosed by laparotomy were taken into consideration.

Extraction and Determination of Putrescine, Spermidine, and Spermine. Tissue samples (0.4 to 1 g, wet weight) were frozen on dry ice and then homogenized in 5 ml 2% perchloric acid (w/v). The homogenate was centrifuged in the cold at 10,000 × g for 15 min, and the precipitate was washed twice with 2.5 ml 2% perchloric acid (13). Polyamine levels were determined on combined supernatants by

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The polyamine concentrations were then corrected for the method of Inoue and Mizutani (13). Polyamines in urine were determined by the method of Fujita et al. (9). In urine assays we occasionally detected on the electrophoretograms a band between those of putrescine and spermidine, which was not taken into consideration in the polyamine determinations.

The recovery of labeled polyamines added to 10 samples of liver and hepatoma homogenates was 95.2 ± 1.5% (S.E.), and that added to urine samples was 97.3 ± 1.2%. The polyamine concentrations were then corrected for these values. Urinary creatinine concentrations were determined by the method of Inoue and Mizutani (13). Polyamine determinations were carried out on large tumors greater than 25 mm in diameter and in tumors about 10 to 20 mm in diameter that developed after 120 and 150 days, respectively, of the carcinogenic diet. A significant increase of spermine was also noted in these tumors. In large tumors more than 25 mm in diameter, which developed after 180 days of the 4-DAB diet, the polyamine concentration decreased, probably in relation to the presence of some areas of necrosis observed at macroscopic as well as at microscopic examination. The spermidine:spermine molar ratio in the liver of rats fed on 4-DAB was higher than that observed in the liver of control rats of the same age; the maximum value (1.62) was noted in tumors 10 to 20 mm in diameter.

**RESULTS**

**Polyamine Concentrations in Rat Liver during 4-DAB Carcinogenesis.** To observe whether the special low-riboflavin diet used normally for 4-DAB administration could modify per se the hepatic levels of polyamines, we determined in preliminary experiments the concentrations of these substances in the liver of rats fed on this special diet (white Miller's diet) and of rats of the same age fed on a normal complete diet (controls). The results showed that the administration of white Miller's diet without carcinogen for 2, 4, and 6 months did not modify the levels of putrescine, spermidine, or spermine in the rat livers. Therefore, the data obtained for rats fed on the 4-DAB diet were compared with those of control rats.

The results reported in Table 1 demonstrate that, in control animals fed on a normal complete diet, putrescine and spermidine levels in liver decreased with increasing age, in accordance with the results of Jānne et al. (15). Spermine levels showed minor changes and decreased only slightly. The spermidine:spermine molar ratio changed with the age of the rat and was 1.65 and 0.89 in 1- and 7-month-old animals, respectively.

In rats fed on the 4-DAB diet (Table 1), the putrescine level significantly increased after only 15 days of the carcinogenic diet, and it reached the maximum value after 90 days when small tumor nodules 2 to 4 mm in diameter were observed in the liver. After 90 days the putrescine level slowly decreased, but it remained significantly higher than did control values. Spermidine concentration significantly increased after 60 days of the 4-DAB treatment, when the livers were still apparently normal, and it remained at this level until the 90th day of the diet, when livers showed small tumor nodules 2 to 4 mm in diameter. Subsequently, a further increase of the spermidine level was observed in small growing tumors about 5 to 8 mm in diameter and in tumors about 10 to 20 mm in diameter that developed after 120 and 150 days, respectively, of the carcinogenic diet. A significant increase of spermine was also noted in these tumors. In large tumors more than 25 mm in diameter, which developed after 180 days of the 4-DAB diet, the polyamine concentration decreased, probably in relation to the presence of some areas of necrosis observed at macroscopic as well as at microscopic examination. The spermidine:spermine molar ratio in the liver of rats fed on 4-DAB was higher than that observed in the liver of control rats of the same age; the maximum value (1.62) was noted in tumors 10 to 20 mm in diameter.

**Polyamine Concentrations and Protein Synthesis Rate in Rat Liver during 4-DAB Carcinogenesis.** Since polyamines may have a role in the regulation of protein synthesis (12, 35, 36), we studied the rate of leucine incorporation into proteins of slices obtained from the same samples of control animals fed on a normal complete diet, putrescine and spermidine levels in liver decreased with increasing age, in accordance with the results of Jānne et al. (15). Spermine levels showed minor changes and decreased only slightly. The spermidine:spermine molar ratio changed with the age of the rat and was 1.65 and 0.89 in 1- and 7-month-old animals, respectively.

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**Table 1**

<table>
<thead>
<tr>
<th>Age of rats (days)</th>
<th>4-DAB feeding (days)</th>
<th>Polyamine concentrations (nmole/g, wet wt)</th>
<th>Spermidine:Spermine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Putrescine</td>
<td>Spermidine</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>44 ± 3⁻</td>
<td>1262 ± 59</td>
</tr>
<tr>
<td>45</td>
<td>15ᵇ</td>
<td>29 ± 2 ⁴</td>
<td>67 ± 5 ⁴</td>
</tr>
<tr>
<td>60</td>
<td>30ᵇ</td>
<td>10 ± 1 ⁴</td>
<td>136 ± 10</td>
</tr>
<tr>
<td>90</td>
<td>60ᵇ</td>
<td>6 ± 1 ⁴</td>
<td>267 ± 20</td>
</tr>
<tr>
<td>120</td>
<td>90ᵇ</td>
<td>3 ± 1 ⁴</td>
<td>382 ± 28</td>
</tr>
<tr>
<td>150</td>
<td>120ᵇ</td>
<td>0</td>
<td>490 ± 18</td>
</tr>
<tr>
<td>180</td>
<td>150ᶠ</td>
<td>0</td>
<td>222 ± 18</td>
</tr>
<tr>
<td>210</td>
<td>180ᵃ</td>
<td>0</td>
<td>202 ± 16</td>
</tr>
</tbody>
</table>

ᵃ Mean ± S.E.
ᵇ Polyamine determinations were carried out on livers.
ᶜ p < 0.01 (Student's t test).
ᵈ Polyamine determinations carried out on livers with small tumor nodules 2 to 4 mm in diameter.
ᵉ Polyamine determinations carried out on tumors 5 to 8 mm in diameter.
ᶠ Polyamine determinations carried out on tumors 10 to 20 mm in diameter.
ᵍ Polyamine determinations carried out on large tumors greater than 25 mm in diameter.
tissue used for the polyamine determinations, and the results were compared with the polyamine levels. Chart 1 shows that, up to 60 days of age, the rate of liver protein synthesis decreased in controls as well as in rats fed on 4-DAB diet. Thereafter, the rate of liver protein synthesis remained almost unchanged up to 210 days of age in controls, whereas it progressively increased in 4-DAB-fed rats. The highest value of protein synthesis was reached in tumors 10 to 20 mm in diameter, which developed in 180-day-old rats after 150 days of the 4-DAB diet. In tumors greater than 25 mm in diameter, which developed after 180 days of the 4-DAB diet, the rate of leucine incorporation into proteins decreased, probably due to the presence of some areas of necrosis as previously mentioned. The results reported in Chart 1 also show that the rate of protein synthesis correlates well with the concentration of total polyamines in normal liver as well as in the liver from rats fed on the 4-DAB diet. In the case of liver carcinogenesis, a correlation was also observed between the rate of protein synthesis (Chart 1) and spermidine and spermine concentrations (Table 1).

**Urinary Polyamines in 4-DAB Hepatoma-bearing Rats.**

![Chart 1. Comparison between the rate of protein synthesis and the concentration of total polyamines in the liver of normal and 4-DAB-fed rats. The treatment with 4-DAB started on the 30th day of age. Protein synthesis was studied on tissue slices (40 to 60 mg, wet weight) incubated for 60 min at 38° in Krebs-Ringer phosphate containing 1.5 µmoles of L-[1-14C]leucine (specific activity, 0.266 mCi/mole). Medium volume, 3 ml; gas phase, air. Polyamine concentrations were measured as described in the text. Each point represents the mean ± S.E. of 8 experiments. The values of total polyamine concentration were calculated from those reported in Table 1. Total polyamines in normal (*) and 4-DAB-fed rats (•); leucine incorporation in normal (Δ) and 4-DAB-fed rats (△).](chart1.png)

The significant increase of polyamines in hepatomas led us to study the levels of these substances in the urine of normal and hepatoma-bearing rats. As reported in Table 2, the total polyamine level in the urine of the animals with hepatomas was significantly greater than that of control rats. The values of putrescine, spermidine, and spermine were, respectively, 2.6, 4.2, and 4.2 times those of the control group. Increases of the same order of magnitude were also observed when urinary polyamine levels were expressed as µg per mg creatinine, as suggested by Durie et al. (6). In this case the data obtained in normal and hepatoma-bearing rats were, respectively, 16.3 ± 2.3 and 42.7 ± 6.4 for putrescine, 9.0 ± 0.5 and 37.6 ± 4.9 for spermidine, and 11.5 ± 1.5 and 48.6 ± 6.8 for spermine.

**DISCUSSION**

Although some authors have reported that oncogenic substances increase polyamine concentrations (20) as well as ornithine decarboxylase (EC 4.1.1.17) and S-adenosylmethionine decarboxylase (EC 4.1.1.50) activities in animal tissues (21, 27), the behavior of polyamines during a process of chemical carcinogenesis has never been studied. The results reported in this paper indicate that polyamines may play a role in the growth of hepatomas and in their protein synthesis.

In our experiments we observed that concentrations of putrescine, spermidine, and spermine were significantly higher in the liver of rats fed on 4-DAB and in the resultant hepatomas in comparison with those of normal livers from control rats of the same strain, sex, and age. These modifications were actually due to the carcinogen, since the special low-riboflavin diet used normally to obtain the carcinogenic effect of 4-DAB (19) did not modify, in the absence of the carcinogen, the polyamine concentrations.

The first change observed during liver carcinogenesis by 4-DAB was an early increase of putrescine concentration, which reached the maximum level (more than 100 times higher than that in controls) when small tumor nodules were present. These results suggest that an early activation of ornithine decarboxylase (the rate-limiting enzyme in the biosynthesis of polyamines) (14, 31) may actually occur after a completely carcinogenic dose of 7,12-dimethylbenz(a)anthracene (21).

**Table 2**

*Urinary polyamine levels in normal and 4-DAB hepatoma-bearing rats*

<table>
<thead>
<tr>
<th>Animals</th>
<th>Putrescine (µg/24 hr)</th>
<th>Spermidine (µg/24 hr)</th>
<th>Spermine (µg/24 hr)</th>
<th>Total (µg/24 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rats</td>
<td>82 ± 12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45 ± 3</td>
<td>58 ± 8</td>
<td>185 ± 22</td>
</tr>
<tr>
<td>Hepatoma-bearing rats</td>
<td>217 ± 33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>191 ± 25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>247 ± 35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>655 ± 92&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± S.E.

<sup>b</sup> p < 0.01 (Student’s t test).
In our experiments the increase of putrescine was followed by the increase of spermidine and spermine concentrations, which reached the maximum levels in growing hepatomas. The increase in spermidine and spermine levels seems to be related to the accumulation of putrescine, which plays a critical role in regulation of the activity of S-adenosylmethionine decarboxylase (7, 11, 22, 38), the rate-limiting enzyme in the synthesis of spermidine and spermine in eukaryotic cells (35, 38). S-Adenosylmethionine decarboxylase activity increased in rat liver after 3-methylcholanthrene and benz(a)pyrene administration (27) and in mouse epidermises after the application of tumor-promoting compounds (21).

In rat liver during 4-DAB carcinogenesis and in the resultant hepatomas, the spermidine:spermine molar ratio was significantly higher than that of the controls, and it was highest in growing hepatomas. This high ratio was based on an increase in the concentration of spermidine and in a simultaneous minor increase in that of spermine. High spermidine:spermine ratios have been reported for other compounds (21). The high concentrations of polyamines in hepatomas were reflected in significantly higher levels of putrescine, spermidine, and spermine in the urine of tumor-bearing rats compared to those of normal rats of the same age. This observation confirms that there is a relationship between tumor metabolism of polyamines and their urinary levels (6).

Polyamines have been shown to stimulate various steps of protein synthesis in vitro, but there is no direct evidence that these substances exert the same effect in vivo. Polyamines stabilize and maintain the association of the ribosomal subunits (1, 35, 36) and promote the attachment of free ribosomes to endoplasmic reticulum membranes (16, 35, 36). Furthermore, these substances stimulate the methylation and the aminocyclation of tRNA as well as the binding of mRNA and aminocycl-tRNA to ribosomes (12, 35, 36). The close correlation observed in this study between polyamine levels and the rate of leucine incorporation into proteins of rat liver during 4-DAB carcinogenesis raises the possibility that polyamines also play a role in tumor protein synthesis in vivo.

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