Changes in the Nucleotide Compositions of Nucleolar 45 S RNA of Azo Dye-induced Hepatoma

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SUMMARY

Many investigators have reported that nucleolar 45 S RNA in its nucleotide composition in various transplantable tumors is similar in that it has less adenylic acid and more cytidylic acid than the 45 S RNA of normal liver. In this work the 45 S RNA of primary, 3'-methyl-4-dimethylaminoazobenzene-induced hepatocellular carcinoma was compared with that of normal liver.

An improved method, which is done with cell sap, was established to obtain undegraded 45 S RNA from tissues with high RNase contents, such as hepatomas involving necrotic regions.

The nucleotide composition of nucleolar 45 S RNA of azo dye-induced primary hepatocellular carcinomas showed the same characteristics as in many other tumors. Nucleolar RNA of nontumor regions, separated from livers which had solid tumors, had a normal nucleotide composition and did not have a low adenylic acid content, although many precancerous nodules could be detected histologically. The nucleotide composition of nucleolar 45 S RNA of the liver of rats fed on 4'-methyl-4-dimethylaminoazobenzene was similar to that of normal liver. Therefore, the difference in the base composition of nucleolar 45 S RNA in hepatomas and in normal liver may reflect a difference between that of cancer and normal tissue. The low adenylic acid content of nucleolar 45 S RNA is a common feature of cancer tissue. The reasons why cancer has this feature are discussed.

INTRODUCTION

It is well known that rapidly labeled nucleolar 45 S RNA is a precursor of ribosomal RNA and that 28 S and 18 S RNA's produced by 45 S RNA are transferred to the cytoplasm after maturation to ribosomal subunits (15, 19, 22, 23, 28). Many authors (2, 13, 17, 27) have reported that the \(^{32}\text{P} \) base composition of nucleolar 45 S RNA of transplantable tumors has a lower adenylic acid content than normal liver, and this seems to be true in various kinds of tumors. On the contrary the base composition of nucleolar 45 S RNA from regenerating rat liver is similar to that of normal liver, not of tumors (12). Therefore, the characteristic base composition of tumors does not simply reflect rapid growth. Only liver however has been used as a control in studies on transplantable tumors, because it is much more difficult to isolate nucleolar 45 S RNA from other normal rat tissues. Since Morris hepatoma, which has many characteristics of normal liver, also has nucleolar 45 S RNA like that of tumor tissue, the possibility that the difference between the base composition of normal liver and cancer tissue might be due to organ specificity is unlikely.

In the present study the hepatoma induced primarily in liver with 3'-Me-DAB usually proved to be a hepatocellular tumor, not a bile duct tumor. It is important that tumors used for biochemical comparison with normal liver should be hepatomas in the sense of having histological affinities to liver parenchyma (21). Although it has been suggested that even supposedly liver cell tumors originate from bile duct cells (20), most of the primary hepatomas used in these experiments were classified as hepatocellular carcinomas. Moreover a comparison between normal liver and the primary hepatoma induced with 3'-Me-DAB was not complicated by the effect of transplantation (21). Thus the nucleolar 45 S RNA in the primary induced hepatoma had a lower adenylic acid content than that of normal liver. Furthermore the base composition characteristic of tumor was observed only in the tumor tissues and not in the precancerous regions.

MATERIALS AND METHODS

Male Sprague-Dawley rats, weighing approximately 100 g, were divided into 3 groups. One group was fed a basal diet purchased from Oriental Food Co., Tokyo; the second was fed a diet containing 0.06% 3'-Me-DAB; and the third group was fed a diet containing 4'-Me-DAB, which has little carcinogenic effect on rat liver. 3'-Me-DAB and 4'-Me-DAB were purchased from Tokyo Kasei Chemical Co., Tokyo. Carrier-free orthophosphate-\(^{32}\text{P} \) was a product of the Japan Atomic Energy Research Institute.

Rats were sacrificed by cervical dislocation either 20 min or 2 hr after injection of orthophosphate-\(^{32}\text{P} \) through the jugular vein. Livers were perfused with ice-cold 0.9% NaCl

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\(^{2}\)The abbreviations used are: 3'-Me-DAB and 4'-Me-DAB, 3'- and 4'-methyl-4-dimethylaminoazobenzene.
solution and then 0.25 M sucrose. The following procedures were carried out at 2—4°. Small tumors, which often developed in the right lobe of the liver at an early stage in carcinogenesis with 3'-Me-DAB, were used. The tumor regions frequently showed necrotic areas, which must have high ribonuclease activity (4). Liver or tumor tissues were chopped thoroughly with scissors and homogenized in 2.25 M sucrose containing 3.3 mM calcium acetate in a homogenizer with a loosely fitting Teflon pestle. Nuclei were prepared according to the method of Chauveau et al. (3). Nuclear pellets were suspended in liver cell sap (1 ml/g, original wet weight) containing 2 mM calcium acetate, which was freshly prepared as described by Shortman (25). Undergraded nucleolar RNA could only be isolated from 3'-Me-DAB-treated liver in this way. Nucleoli were isolated after sonic disruption (16). Nucleolar RNA was extracted by the sodium dodecyl sulfate-phénol method (7, 29), carrier yeast RNA was added, and the nucleolar RNA was precipitated by overnight storage at -20° with 2 volumes of 90% ethanol containing 2% potassium acetate. The RNA was collected by centrifugation and dissolved in a small volume of 0.05 M sodium acetate buffer, pH 5.0.

Sucrose density gradient sedimentation and determination of radioactivity were carried out as described previously (7). Nucleotide analysis was carried out according to the method of Hurlbert et al. (8). For removal of contaminating inorganic phosphates, if present, all 4 mononucleotide fractions were treated with triethylamine as described by Sugino and Miyoshi (31).

RESULTS

Pattern of Nucleolar RNA in Sucrose Density Gradient Sedimentation. When nuclear pellets were suspended in 0.25 M sucrose, nucleolar RNA was degraded (Chart 1B) during sonic disruption (26), especially when nuclei had been obtained from the liver of rats treated with 3'-Me-DAB (24). Thus, as described in "Materials and Methods," nuclear pellets were suspended in freshly prepared liver cell sap from normal rat liver (25). Unless this liver cell sap contained 2 mM calcium acetate, nuclei were disrupted during sonic disruption. The RNA extracted from nucleoli prepared in this way gave a better pattern on a sucrose density gradient, i.e., with a large amount of rapidly sedimenting RNA (Chart 1A).

Essentially, there was no difference between the patterns of nucleolar RNA prepared from rats on a normal diet and those from rats on diets containing 4'-Me-DAB or 3'-Me-DAB. The peak of radioactivity in 45 S RNA was predominant after short pulse labeling, but after 120 min the amount of radioactive 28 S RNA had increased and exceeded the amount of 45 S RNA (15). Chart 1A represents the pattern of sucrose density gradient sedimentation of nucleolar RNA of hepatoma after 120 min.

Nucleotide Compositions of Subfractionated Nucleolar RNA. As shown in Table 1, nucleolar 45 S RNA of 3'-Me-DAB-induced hepatoma, which was confirmed histologically to be mainly a hepatocellular carcinoma, had a nucleotide composition similar to that of other cancer tissues, i.e., the content of adenylic acid was very low. Since the tumor regions were contaminated with normal liver cells which could be detected microscopically, the characteristic pattern of $^{32}$P distribution of the tumor was somewhat diluted. However, the nucleotide composition of nucleolar RNA of noncancerous regions, which were separated from the same tumor-carrying livers, was different from that of tumors. The nontumorous regions were examined microscopically and found to have many precancerous features, such as hyperplastic nodules. The nucleotide ratio of nucleolar RNA of rats which had been fed on a diet containing 4'-Me-DAB for 4 to 6 months was similar to that of normal rats. Thus, the lower adenylic acid content of tumor nucleolar RNA is not due to the toxicity of the aminoazo dyes, but reflects the malignant transformation of normal liver cells to hepatoma cells.

Nucleolar 28 S RNA of tumor tissue had the same difference from normal in base composition as nucleolar 45 S RNA.
Table 1

Nucleotide compositions of subfractions of nucleolar RNA

Animals were fed a basal diet (Oriental Food Co., Tokyo, Japan) with or without 3'-Me-DAB or 4'-Me-DAB. Rats were treated by injection via the jugular vein with 2 mCi orthophosphate-32P and were examined 2 hr later. Nucleoli were isolated and nucleolar RNA was extracted as described in "Materials and Methods." Nucleotide compositions are expressed as percentages of the total counts in each nucleotide. Each value is the average of 3 to 4 experiments. Standard errors were calculated by the equation: S.E. = \sqrt{\frac{\sum(x - \bar{x})^2}{n(n - 1)}}

<table>
<thead>
<tr>
<th>Nucleotide Acid</th>
<th>45 S RNA 3'-Me-DAB Tumor region</th>
<th>45 S RNA 3'-Me-DAB Nontumor region</th>
<th>28 S RNA 4'-Me-DAB Tumor region</th>
<th>28 S RNA 4'-Me-DAB Nontumor region</th>
<th>Normal</th>
<th>A+U/G+C^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytidyl acid</td>
<td>29.8 ± 1.2</td>
<td>26.7 ± 1.1</td>
<td>26.3 ± 0.8</td>
<td>26.0 ± 0.8</td>
<td>28.0 ± 0.8</td>
<td>0.49</td>
</tr>
<tr>
<td>Adenylic acid</td>
<td>14.6 ± 0.6</td>
<td>17.9 ± 0.5</td>
<td>18.2 ± 0.3</td>
<td>18.5 ± 0.4</td>
<td>18.5 ± 0.4</td>
<td>0.58</td>
</tr>
<tr>
<td>Guanylic acid</td>
<td>37.2 ± 0.8</td>
<td>37.3 ± 0.6</td>
<td>38.4 ± 0.8</td>
<td>35.7 ± 0.4</td>
<td>37.6 ± 1.2</td>
<td>0.59</td>
</tr>
<tr>
<td>Uridyl acid</td>
<td>18.4 ± 0.1</td>
<td>18.1 ± 0.5</td>
<td>17.3 ± 0.6</td>
<td>18.1 ± 1.8</td>
<td>17.3 ± 0.2</td>
<td>0.55</td>
</tr>
</tbody>
</table>

^aC, cytidylic acid; A, adenylic acid; G, guanylic acid; U, uridylic acid.

Effect of the Period of Azo Dye Feeding. Since most of the radioactivity was located in the 28 S, 35 S, and 45 S regions, and since both 28 S and 45 S RNA's had similar abnormal nucleotide compositions (Table 1), the total nucleolar RNA was used to study the change of nucleolar RNA in the course of carcinogenesis.

The nucleotide compositions of total nucleolar RNA of livers in which no solid tumor could be seen macroscopically, even after animals had been fed with 3'-Me-DAB for 3 to 6 months, were similar to those of normal livers, although there were many histological changes in the liver after long-term administration of 3'-Me-DAB (4, 5, 11). The proportion of parenchymal cells to bile duct cells also changed during the period of azo dye feeding (4, 30). This indicates that neither 3'-Me-DAB itself nor precancerous regions caused the lower adenylic acid and higher cytidyl acid contents.

DISCUSSION

The main purpose of this work was to study whether the nucleolar 45 S RNA of primary hepatoma induced by 3'-Me-DAB had characteristics similar to those of many other tumors and, if so, what were the tumor-like characteristics in the course of carcinogenesis by azo dyes.

An improved method to obtain intact nucleolar 45 S RNA was established with the use of rat liver supernatant and calcium ions during isolation of nucleoli. Increased RNase activity is usually observed not only after sonic disruption of nuclei (26) but also in the course of carcinogenesis by 3'-Me-DAB (24). It has been reported that rat liver supernatant can be used as an RNase inhibitor (1, 9, 10) to obtain intact polysomes. Our workers have found that calcium ions (2 mM final concentration) were necessary in rat liver supernatant to maintain the integrity of nucleoli. The sedimentation profiles of nucleolar RNA from liver of rats fed with 3'-Me-DAB were much improved by utilizing rat liver supernatant.

In this study, hepatomas which were primarily induced by 3'-Me-DAB and not transplanted, i.e., those which developed in liver, were used to examine common features of nucleolar 45 S RNA in cancers. Parallel biochemical and histological studies were made, and regions containing mainly hepatocellular carcinomas were used in the present study, although some contaminating normal liver cells were detected in our tumor preparations. It was found by 32P nucleotide analysis that nucleolar 45 S RNA of hepatomas induced by 3'-Me-DAB had lower adenylic acid and higher cytidyl acid contents than those of normal liver.

The same difference between the base compositions of nucleolar 45 S RNA in normal and tumor tissue has been seen with transplantable tumors (17, 27), minimal deviation hepatomas (2, 13), and hepatomas induced by carcinogenic azo dyes. This difference can be shown only by 32P base analysis, and not by ultraviolet analysis (14, 27). There are several possible explanations for the difference between the 32P nucleotide compositions of nucleolar 45 S RNA of tumors and liver.

First, there may be 2 or more species of 45 S RNA in the nucleoli that are present in the same relative amounts of tumor and liver tissue. Thus, the same base composition

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3Since the basal diet purchased from Oriental Food Co., contains 24% protein, this relatively high protein content might be responsible for the low incidence (approximately 30 to 40% after 6 months) of hepatomas with 3'-methyl-4-dimethylaminoazobenzene.
Table 2

Effect of 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) diet on nucleolar RNA of rat liver

Rats were fed 3'-Me-DAB for the periods indicated. Livers with no visible tumor were used for this experiment. Animals were given injections of 2 mc of orthophosphate-32P and examined 20 min later. 32P analysis of nucleotides were made on total nucleolar RNA. Each value is the average of 3 to 4 experiments.

<table>
<thead>
<tr>
<th>No. of mo.</th>
<th>C*</th>
<th>A</th>
<th>G</th>
<th>U</th>
<th>A+U/G+C</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>27.4 ± 0.6</td>
<td>18.8 ± 0.3</td>
<td>36.1 ± 0.5</td>
<td>17.8 ± 0.4</td>
<td>0.58</td>
</tr>
<tr>
<td>4</td>
<td>28.7 ± 0.4</td>
<td>17.2 ± 0.0</td>
<td>35.7 ± 0.4</td>
<td>18.6 ± 1.5</td>
<td>0.56</td>
</tr>
<tr>
<td>5</td>
<td>27.2 ± 0.7</td>
<td>17.8 ± 0.7</td>
<td>36.9 ± 0.6</td>
<td>18.6 ± 0.3</td>
<td>0.56</td>
</tr>
<tr>
<td>6</td>
<td>27.2 ± 0.4</td>
<td>17.2 ± 0.1</td>
<td>35.2 ± 0.4</td>
<td>20.4 ± 0.5</td>
<td>0.60</td>
</tr>
</tbody>
</table>

*C: cytidylic acid; A, adenylic acid; G, guanylic acid; U, uridylic acid.

would be found by ultraviolet analysis. However, if the rates of synthesis of these species differed, then one which was strongly labeled in tumor tissues containing less adenine and more cytidine could cause the difference observed by 32P base analysis. The next possibility is that different multiple species of nucleolar 45 S RNA are present in different tissues. If the rapidly labeled 45 S RNA of tumors is different from the 45 S RNA of liver, and this RNA moves out much more rapidly from the nucleoli than the bulk of the nucleolar 45 S RNA, the discrepancy between the 32P and the ultraviolet analyses can be explained. Although 45 S nucleolar RNA has been partially fractionated (27), it is uncertain whether 45 S RNA is a single molecular species or whether it consists of 2 or more species of RNA. The third possibility is that the 45 S RNA obtained from liver is contaminated by trace amounts of some highly labeled RNA rich in adenosine (6) which sediments with the 45 S RNA components. In support of this possibility, Pene et al. (18) recently reported that low-molecular-weight RNA was present in 28 S RNA and was probably bound by hydrogen bonds.

There may be many other possible explanations as to why tumor tissues have this peculiar feature in common. However, it is important to know what 45 S RNA is and what kinds of RNA are involved in the 45 S region before speculating further about 45 S RNA.

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REFERENCES


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