Origin of Increased Deoxycytidine Excretion into Urine of Rats Bearing Yoshida Ascites Sarcoma

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ABSTRACT

The metabolism of deoxycytidine (dCyd) and dCyd nucleotides in Yoshida ascites sarcoma (YS) cells and the host rat liver was investigated with reference to the increased excretion of urinary dCyd.

Incorporation of [14C]orotic acid into the livers of rats at the fifth day after the transplantation of YS cells, the time when the amount of excretion of dCyd in urine was near maximal, was 2 times higher than that into the normal rat livers. After the injection of [14C]orotic acid, the ratio of the specific radioactivity of cytidine diphosphate reducense (EC 1.17.4.1), in YS were higher than those in both rat ascites hepatoma AH 7974 and Walker 256 carcinosarcoma, the transplantations of which did not induce increased excretion of dCyd in urine of the hosts. The activities of cytidine triphosphate synthetase (EC 6.3.4.2) and cytidine diphosphate reducense (EC 3.5.4.5) in YS cells were lower than those in the other two tumors investigated. The activities of cytidine triphosphate synthetase and cytidine diphosphate reducense in the livers of YS-bearing rats were elevated compared with those in the livers of rat ascites hepatoma AH 7974- or Walker 256 carcinosarcoma-bearing rats and normal rats, while the activities of dCyd kinase, 5'-nucleotidase (EC 3.1.3.5), and dCyd deaminase were similar between normal rat livers and tumor-bearing rat livers.

These results suggest that the increased excretion of urinary dCyd in YS-bearing rats could be caused by both the stimulation of the synthesis of dCyd nucleotides and the low activity of dCyd deaminase in YS cells as well as in the host liver.

INTRODUCTION

Abnormal excretion of some of the nucleic acid constituents, such as methylated purines and pyrimidine nucleosides, has been observed in urine of cancer patients as well as of experimental animals bearing certain tumors (1, 12, 16, 18, 22, 29, 33). However, the detailed mechanism of this excretion has not been well elucidated.

In our previous paper (29), it was shown that YS-bearing rats excreted significantly higher quantities of dCyd, pseudouridine, and cytidine in urine than did normal rats. We proposed that the increase of urinary dCyd and pseudouridine could be derived from not only the tumor cells but also the host liver, since the contribution of the radioactivity of injected [6-14C]OA to these nucleosides was higher than that of either [4-14C]uridine or [U-14C]cytidine. This proposal was based on the fact that the radioactivity of [14C]OA was preferentially incorporated into the host liver than into the tumor cells (14, 17, 29).

In tumor-bearing animals, a number of biochemical and biological alterations have been observed in not only the liver but also other organs. Among the organs, the liver is known to exhibit the most significant changes for animals as described before (3, 4, 20, 29, 30).

We postulated in the previous paper that the increase of urinary dCyd is caused by the stimulated biosynthesis of dCyd nucleotides rather than by the enhanced degradation of DNA, since thymidine and 5-methyl-dCyd, which are pyrimidine nucleosides of DNA, could never be detected in urine of YS-bearing rats (29).

In this study, in order to confirm a possibility that the host liver is playing a role in the increase of dCyd excreted in urine of YS-bearing rats, the incorporation of [14C]OA into the host liver and loss of radioactivities from the host liver after the tumor transplantation were investigated and compared with those of control rat livers. Furthermore, we report the changes in the activities of enzymes relating to the metabolism of dCyd and dCyd nucleotides in both YS cells and the host liver.

MATERIALS AND METHODS

Materials. [6-14C]OA (49.1 mCi/mmol), [2-14C]UTP (46.8 mCi/mmol), [U-14C]CDP (435 mCi/mmol), and [2-14C]dCyd (29.7 mCi/mmol) were purchased from New England Nuclear, Boston, MA. Avicel SF cellulose and polyethyleneimine cellulose thin-layer plates were obtained from Funakoshi Pharmaceutical Co., Tokyo, Japan, and E. Merck GmbH, Darmstadt, German Federated Republic, respectively.

Tumora. YS was maintained by serial transplantation every 5 or 6 days via i.p. injection of 0.5 ml of the ascites fluid into 7-week-old male Donryu rats (Matsumoto Experimental Animal Co., Chiba, Japan) (29). AH 7974 was also maintained by serial transplantation every 7 days via i.p. injection of 0.1 ml of the ascites fluid into 7-week-old male Donryu rats. Walker 256 was maintained by serial transplantation every 7 days via i.m. injection of 20% crushed solid-tumor suspension in 0.9% NaCl solution into the right thigh of 6-week-old male Wistar/Ibmichi rats (Matsumoto Experimental Animal Co.).

Administration of [14C]OA into YS-Bearing Rats and Measurement of Radioactivity in the Liver. To determine the incorporation of [14C]OA, rats were given injections i.p. of [14C]OA at a dose of 10 μCi/200 g body weight 5 days after the transplantation of YS, the time when

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the amount of excretion of dCyd in urine was near maximal. Thirty min later, rats were sacrificed by decapitation, and the excised liver and harvested tumor cells were homogenized with 5 volumes of cold 10% perchloric acid to prepare acid-soluble RNA and DNA fractions according to the method of Schmidt and Thannhauser (27). Radioactivities in each fraction were measured with 10 ml of Aquasol 2 (New England Nuclear) using a Beckman LS-250 liquid scintillation counter. In order to examine the pattern of RNA labeling, 3 rats in each experimental group were used in both YS-bearing rats (4 days after the tumor transplantation) and normal rats. Rats were given injections of \(^{14}C\)OA and sacrificed 1, 6, and 24 hr later. RNA was isolated from livers excised from both YS-bearing rats and normal rats and harvested from the tumor cells according to the Kirby method modified by Roberts and Warwick (25) and then hydrolyzed in 0.3 N KOH at 37° for 18 hr. The hydrolysates were applied onto a Whatman No. 3MM filter paper and chromatographed in Solvent System I consisting of 2-propanol/concentrated HCl/water (70/15/15, v/v/v). The areas corresponding to cytidylic and uridylic acids were cut out, and these nucleotides were extracted with 0.01 M HCl. The amounts of both nucleotides were calculated from total UV absorbance and molar extinction coefficients at pH 2 (5), and radioactivities were measured with 10 ml of Bray’s solution.

**Administration of \(^{14}C\)OA into Rats Prior to the Tumor Transplantation and Measurement of Radioactivities of Urinary Nucleosides and the Liver.** Rats, weighing 80 to 100 g, received a total amount of 20 \(\mu\)Ci of \(^{14}C\)OA, which was injected i.p. every other day, 5 \(\mu\)Ci at each time. YS cells were transplanted into the rats 13 days after the first injection of \(^{14}C\)OA. The rats without the tumor transplantation were used as control. All rats were kept individually in metabolic cages, and urine was collected every 24 hr between the third and fifth days after the tumor transplantation. Quantification of urinary dCyd and pseudouridine as well as measurement of radioactivities of each compound was carried out as described in our previous paper (29). After collection of urine, the rats were killed, and their livers were excised. Thereafter, acid-soluble RNA and DNA fractions were obtained by the method of Schmidt and Thannhauser (27). Radioactivities were measured as described above.

**Enzyme Preparation.** Rats were killed by cervical dislocation 5, 7, and 8 days after the transplantation of YS, AH 7974, and Walker 256, respectively. The harvested ascites tumor cells and the crushed solid tumors were washed with cold 0.9% NaCl solution to remove erythrocytes. The washed tumor cells and the excised livers perfused with cold 0.9% NaCl solution were homogenized with 0.25 m sucrose containing 0.05 M Tris-HCl (pH 7.5) and 0.01 M 2-mercaptoethanol using Polytron (Kinematica GmbH, Luzern, Switzerland) and centrifuged at 105,000 x g for 60 min at 4°. The clear supernatants were used for the assay of CTP synthetase, dCyd kinase, and 5'-nucleotidase activities. For the assay of CDP reductase and dCyd deaminase activities, the fraction precipitated from the above supernatants between 0 and 40% saturation with ammonium sulfate was used after dialysis against 0.05 M Tris-HCl (pH 6.8), 10 mM MgCl\(_2\), 100 mM Tris-malate (pH 6.5), and an aliquot of the enzyme preparation. The mixture was incubated at 37° for 30 min and terminated by heating at 100° for 1 min. After centrifugation, 20 \(\mu\)l of the supernatant were applied onto a polyethyleneimine cellulose plate with authentic dCDP and dCMP as carriers and chromatographed in Solvent System II (1 mM ammonium acetate/95% ethanol, 75/30, v/v) saturated with boric acid. The radioactivity of dCMP produced was measured as described above.

5'-Nucleotidase activity was assayed according to the method of Naito and Tushima (21). The assay mixture (0.5 ml) contained 3 \(\mu\)l dCMP, 10 \(\mu\)l MgCl\(_2\), 100 \(\mu\)l Tris-malate (pH 6.5), and an aliquot of the enzyme preparation. The mixture was incubated at 37° for 30 min, and the reaction was terminated by adding 0.5 ml of 10% trichloroacetic acid containing 10% Norit A. After removal of precipitated protein and Norit A by filtration, liberated Pi was measured by the method of Fiske and SubbaRow (34).

dCyd deaminase activity was assayed by the method of Wan and Mak (34) with some modifications. The assay mixture (50 \(\mu\)l) contained 10 \(\mu\)M Tris-HCl (pH 7.5), 4 \(\mu\)M \([2-\text{14C}]\)dCyd (0.5 \(\mu\)Ci/\(\mu\)mol), and an aliquot of the enzyme preparation. The mixture was incubated at 37° for 30 min, and the reaction was terminated by adding 10 \(\mu\)l of concentrated HCl. After removal of precipitated protein, 20 \(\mu\)l of the supernatant were applied onto an Avicel SF thin-layer plate with authentic dCyd and dUDP as carriers and chromatographed in Solvent System I. The radioactivity of dUDP produced was measured as described above.

**RESULTS**

Incorporation of Radioactivity into the Liver and Ratio of Specific Radioactivity in Cytidylate to Uridylate Moieties of RNAs. In order to investigate the alteration, if any, of RNA and DNA metabolism in the livers of YS-bearing rats, \(^{14}C\)OA was injected i.p. 5 days after the tumor transplantation, and rats were sacrificed 30 min later. As shown in Table 1, the radioactivities (cpm/g, wet weight, of tissue) in the whole homogenate, acid-soluble, and RNA fractions of the host liver were about 2 times higher than those of the normal rat liver, but this difference was not observed in the DNA fraction. Although the amount of \(^{14}C\)OA taken up by the tumor cells was less than that of the host liver as observed before (29), the ratio of the radioactivities of RNA or DNA to that of the whole homogenate in YS cells was higher than the ratio in the host liver.

Radioactivities incorporated for 1, 6, and 24 hr after the injection of \(^{14}C\)OA into cytidylate to uridylate moieties of liver RNA were measured for both the tumor-bearing rats and normal rats. As shown in Table 2, the ratio of the specific radioactivity of cytidylate to uridylate moieties in host liver RNA was 2 to 3 times higher than that in normal rat liver RNA for all the periods of investigation and became similar to that of YS cell RNA 24 hr after \(^{14}C\)OA injection.
Radioactivities in acid-soluble RNA and DNA fractions of the livers of YS-bearing and normal rats after injection of \([^{14}C]OA\)

\([^{14}C]OA\) was injected i.p. at a dose of 10 \(\mu Ci/200\text{-g body weight}\) into rats 5 days after the transplantation of YS cells. After 30 min, fractionation of the excised livers and harvested tumor cells was carried out as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Whole homogenate</th>
<th>Fractions (cpm (\times 10^{-4}/g), wet wt, of tissue)</th>
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<tbody>
<tr>
<td>YS-bearing rat liver</td>
<td>291.80 (\pm 0.39^a)</td>
</tr>
<tr>
<td>Normal rat liver</td>
<td>137.15 (\pm 40.86)</td>
</tr>
<tr>
<td>YS cells</td>
<td>7.16 (\pm 2.62)</td>
</tr>
</tbody>
</table>

\(^a\)Mean \(\pm S.D.\) of 4 rats.

Urinary dCyd Excretion and Role of Tumor Host Liver

Table 1

Labeling of Urinary dCyd and the Radioactivity Remaining in the Liver after the Tumor Transplantation in Rats Given Injections of \([^{14}C]OA\) in Advance. \([^{14}C]OA\) was administered i.p. at a dose of 20 \(\mu Ci/\text{head}\) into rats in advance. Rats were divided into 2 groups; one group was transplanted with YS cells 13 days after the initial injection of \([^{14}C]OA\), and another group without the tumor transplantation was used as control.

In Table 3 are shown the specific radioactivities of urinary dCyd and pseudouridine at the third, fourth, and fifth days after the tumor transplantation. The specific radioactivities of urinary dCyd showed a tendency to increase, while those of pseudouridine continued to decrease. Only a trace amount of urinary dCyd was found in control rats urine (29).

The radioactivities remaining in the livers of YS-bearing rats in comparison with the corresponding values of the control rat livers are shown in Table 4. The radioactivities (cpm/g, wet weight, of liver) in the whole homogenate of YS-bearing rat livers were about one-half of those of the control rat livers. Moreover, the radioactivities in all the fractions of YS-bearing rat livers were less than those in the control rat livers. The total radioactivity in the whole homogenate of YS cells harvested 5 days after the tumor transplantation was 4200 cpm, which corresponded to only 3.4% of that of the host liver.

Activities of Pyrimidine Metabolic Enzymes. The enzyme activities which play the strategic role in the dCyd metabolic pathway were increased in not only YS and its host liver but also AH 7974 or Walker 256, the transplantation of which did not cause any increased excretion of urinary dCyd (data not shown). All activities of enzymes assayed were linear for at least 30 min and proportional to the enzyme concentration used. The results are summarized in Table 5. The activities of enzymes of pyrimidine nucleotide biosynthesis, such as CTP synthetase, CDP reductase, and dCyd kinase, were very low or scarcely detected in the normal rat liver. The activities of CTP synthetase and CDP reductase increased in the order of YS, AH 7974, and Walker 256, and these activities in YS were 2 to 7 times higher than those in AH 7974 or Walker 256. On the contrary, the activity of dCyd kinase was 2 times lower in YS than in AH 7974. In the livers of the tumor-bearing rats, the activities of CTP synthetase and CDP reductase in the livers of YS-bearing rats were significantly elevated compared with those in normal rat liver, while the increase in the other 2 tumor-bearing rat livers was marginal.

The activities of pyrimidine catabolic enzymes, such as 5'-nucleotidase and dCyd deaminase, in all of the tumor-bearing rat livers were similar to that of the normal rat liver. YS contained a higher activity of 5'-nucleotidase than did AH 7974 and the
normal rat liver. However, the activity of dCyd deaminase in YS was only one-fifteenth and one-twentieth of those in AH 7974 and Walker 256, respectively.

**DISCUSSION**

It has been considered that methylated purines in urine of cancer patients and animals bearing experimental tumors originate from their tumor tissues (2, 31). We proposed previously that dCyd excreted in urine of YS-bearing rats might derive from not only YS cells but also the host liver (29). This study presented the evidences that at least a part of dCyd excreted in urine of YS-bearing rats originated from their livers.

It is noteworthy that the tumor transplantation into rats induced the enhanced turnover of nucleic acids and nucleotides in YS-bearing rat liver (Tables 1, 2, and 4). Thirty min after the injection of [14C]OA into YS-bearing rats, the radioactivities in both the acid-soluble and RNA fractions in the whole homogenate of the host liver were higher than that of the normal rat liver (Table 1), suggesting the stimulation of both OA metabolism and RNA synthesis in the host liver. When the radioactivities of [14C]OA incorporated into cytidylate and uridylyl moieties of RNA of the host liver and the normal rat liver were measured, the ratio of the specific radioactivity of cytidylate to uridylyl moieties of host liver RNA was significantly higher than that of normal rat liver RNA and similar to that of YS cell RNA 24 hr after [14C]OA injection (Table 2). This result indicated that the stimulation of CTP synthesis took place in the host liver and in YS cells. In fact, the activities of CTP synthetase and CDP deaminase were higher than those in the normal rat liver and AH 7974, Walker 256, and their host livers (Table 5). The activities of these enzymes on dCyd metabolism in tumors, tumor-bearing rat livers, and normal rat livers are shown in Table 5.

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*Mean ± S.D. of 4 to 6 determinations.

ND, not determined.

It has been postulated that the increase of urinary dCyd could be explained by the elevated conversion to cytidine and dCyd nucleotides via the salvage pathway from the accelerated degradation products of nucleic acids, mainly of RNA which had been labeled by [14C]OA prior to the tumor transplantation. In fact, the rate of disappearance of the radioactivities from the acid-soluble RNA and DNA fractions of the rat livers was accelerated after the tumor transplantation, compared with that of the control rat livers (Table 4). Although the relative loss of the radioactivity in the DNA fraction of the host liver was more accelerated than that in the RNA fraction (Table 4), the actual amount of loss in the DNA fraction was negligible compared with that in the RNA fraction. This conclusion is based on the fact that the amount of radioactivities in the RNA fraction was several hundred times higher than that in the DNA fraction when YS-bearing rats were given injections of [14C]OA (Table 1). However, this increase of the specific radioactivity of urinary dCyd should be transitory, and its specific radioactivity might decrease consequently, as in the case of urinary pseudouridine (Table 3), since the labeled nucleotides could be diluted with the newly synthesized unlabeled ones.

The day when the maximum peak of the nucleoside excretion in urine of rats after the tumor transplantation was observed varied from one rat to another, depending on the conditions of each rat as described previously (29). In addition, since the rats used in these experiments (Tables 3 and 4) had been labeled with [14C]OA 13 days prior to the tumor transplantation, it was conceivable that another factor caused fluctuation of experimental data. Therefore, the results of only 2 of 3 cases are presented as examples to show the patterns of the changes of the specific radioactivities of urinary nucleosides in Table 3 and those of the changes of loss of the radioactivities in the liver in Table 4.

It has been postulated that the increase of urinary dCyd in YS-bearing rats is due to the stimulation of the synthesis of pyrimidine nucleotides, especially the formation of CTP and dCDP from UTP and CDP, respectively (29). Therefore, it is worthwhile to investigate the enzyme activities which are responsible for the metabolism of dCyd in both YS cells and its host liver and to compare them with those in AH 7974, Walker 256, and the livers of their tumor-bearing rats which did not show any increase of urinary excretion of dCyd as described above.

The activities of CTP synthetase and CDP reductase in YS cells were higher than those in the normal rat liver, AH 7974, Walker 256, and their host livers (Table 5). The activities of these enzymes on dCyd metabolism in tumors, tumor-bearing rat livers, and normal rat livers are shown in Table 5.

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enzymes in the livers of YS-bearing rats were also elevated, compared with that of the normal rat liver, and were higher than those of the livers of AH 7974- and Walker 256-bearing rats. dCyd kinase activities in YS cells and AH 7974 were remarkably high, compared with that in the normal rat liver, although there was no increase in their host livers. However, this activity in YS cells was only one-half of that in AH 7974. Montpeller and Fischer (19) and Kessel (13) reported that dCyd nucleotides are potent inhibitors of dCyd kinase. We have observed that the pool size of dCTP in YS cells was severalfold larger than those in AH 7974 and Walker 256 (data not shown). Therefore, it is conceivable that the conversion of dCyd to dCMP in YS cells might be suppressed, which results in accumulation of dCyd. 5’-Nucleotidase activities which were assayed only with dCMP as a substrate were a little higher in both YS cells and its host liver than in the normal rat liver, while those in AH 7974 and its host liver were similar to those in the normal rat liver. Although this enzyme activity in tumor tissues was usually found to be lower than those in normal tissues (24), Ip and Dao (11) reported high 5’-nucleotidase activity in breast cancer patients. The high activity of 5’-nucleotidase in YS cells could also partly explain the reasons of the dCyd accumulation in YS-bearing rats.

dCyd deaminase activity in YS cells was significantly higher than that in the normal rat liver. However, this activity in YS cells was very low, compared with those in AH 7974 and in Walker 256, the transplantation of which did not induce the increase of urinary dCyd excretion. This result indicates that the accumulation of dCyd could occur in YS cells because dCyd cannot be deaminated sufficiently. Although the increased excretion of urinary dUr was expected in AH 7974- and Walker 256-bearing rats because of the high activity of dCyd deaminase, dUr was not detected in urine. It is possible that dUr is metabolized further. The livers of YS-bearing rats could also contribute to elevated dCyd excretion, because of their potency to supply more dCyd nucleotides than those of AH 7974- and Walker 256-bearing rats. Furthermore, dCyd deaminase activities in all of the tumor-bearing rat livers were similar to that of the normal rat liver.

The enzyme activities for dCyd degradation shown in Table 5 were not high enough to explain the lower amount of radioactivities remaining in the host liver which was observed (Table 4). However, the results shown in Table 4 clearly reveal the accelerated degradation of host liver RNA. Therefore, the possibility that the activities of other degradation enzymes such as RNase in the host liver are enhanced cannot be excluded.

The appearance of YS cells in blood and the invasion into the portal region in YS-bearing rats are known to occur. However, the possibility that enhanced activities of CTP synthetase and CDP reductase in the host liver are due to the contamination of the enzymes of YS cells can be excluded by the following reasons. (a) The portal region is carefully eliminated, and the liver is thoroughly perfused with 0.9% NaCl solution before the preparation of the enzyme extract. (b) YS cells cannot be disrupted under the conditions to prepare the homogenate of the liver. (c) As shown in Table 5, dCyd kinase activity in the host liver was significantly low, compared with that in YS cells. Because the stimulation of the synthesis of cytidine and dCyd nucleotides was observed also in AH 7974, Walker 256, and their host livers, the stimulation might be generalized in other tumors and their host livers. The low activity of dCyd deaminase in YS cells is unique, since dCyd deaminase activities in other tumors used in this experiment and in the mouse ascites hepatoma 129 (28) were very high.

In conclusion, we propose that the increased excretion of dCyd in urine of YS-bearing rats is due to the alterations of the pyrimidine metabolism in not only YS cells but also the host organs, particularly in the liver.

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