Serum Pseudouridine as a Biochemical Marker in the Development of AKR Mouse Lymphoma

Tommaso Russo, Alfredo Colonna, Francesco Salvatore, Filiberto Cimino, Sandra Bridges, and Corrado Gurgo


ABSTRACT

Pseudouridine is a modified nucleoside derived from the degradation of some species of RNA, primarily transfer RNA, the level of which is elevated in biological fluids of tumor-bearing subjects. In order to study the relationship between pseudouridine levels and the development and progression of neoplasia, we have measured pseudouridine levels in the serum of inbred mice with high (AKR) and low (BALB/c) incidence of spontaneous lymphoma and in mice carrying transplantable lymphoid tumors.

Our results show that the serum level of pseudouridine: (a) in healthy mice, is higher in females than in males; (b) increases significantly in female AKR mice in the period preceding the development of lymphoma (preneoplastic period occurring at about 6 months of age); and (c) is highest in AKR mice with lymphoma, the most elevated levels being found in mice with widely disseminated disease. The latter observation was confirmed by experiments with a transplantable AKR lymphoma (T2), where a positive correlation between tumor burden and pseudouridine levels was found. On the contrary, in BALB/c mice carrying a transplantable myeloma tumor (MOPC-460), no increase was seen despite the presence of a considerable tumor burden. The increase of pseudouridine in the preneoplastic period, in the absence of overt disease, is viewed as an early sign of the development of the disease.

INTRODUCTION

Numerous studies have documented the occurrence of increased levels of modified nucleosides in the biological fluids of cancer patients (3, 5, 9, 17, 21, 23–27, 29). Of these nucleosides, pseudouridine is the most frequently and most significantly elevated (5, 21, 23).

We recently demonstrated that serum levels of pseudouridine in patients with several different types of tumors correlated with both the stage of disease and the response to therapy (23). Similar results were reported in other studies which measured urinary levels of pseudouridine in breast cancer patients (25) and in patients with a variety of lymphoid tumors (21).

An increased turnover of tRNA subpopulations has been found in tumor tissues (1), and this has been proposed as a mechanism by which the elevated levels of modified nucleosides are generated. Elevated activity of some tRNA-modifying enzymes (2, 8, 18, 22) has been described in tumor tissues; however, when the tRNA from these tissues was examined, it was found to be hypomodified in most cases (2, 19, 21).

In this article, we report the results of our studies relating pseudouridine levels and the development and progression of neoplasia in an animal system. We have used mice of the AKR strain which spontaneously develop a thymic lymphoma. The AKR lymphoma shares several analogies with human acute lymphoblastic leukemia (20) and has been used as a model for the evaluation of chemotherapeutic regimens (28). The disease occurs with high incidence after the sixth month of age and progresses through 3 distinct stages: Stage 1, the "preleukemic" or preneoplastic stage in which a variety of changes occur in the thymus, including the generation of recombinant retroviruses which are believed to be the etiological agents of the disease (6, 16), and during which no transplantable tumor cells can be detected; Stage 2, the localized stage in which tumor cells are present, but only in the thymus; and Stage 3, the disseminated stage in which widespread involvement of lymphoid and nonlymphoid organs occurs. Thus, the system offers the possibility of assessing changes in the biochemical marker pseudouridine during the transition period, as well as during the progression of the disease. Herein we describe our studies with spontaneous AKR lymphoma and with 2 transplantable lymphoid tumors.

MATERIALS AND METHODS

Mice. AKR and BALB/c mice were from Jackson Laboratory mice and Charles River mice, respectively.

Serum Preparation. Blood was collected by cardiac puncture from mice under ether anesthesia. It was allowed to clot at room temperature for a maximum of 1 hr and was then left overnight at 4° before centrifugation. All samples were kept frozen at -20°C until further processed.

Pseudouridine Determination. Serum pseudouridine was purified and determined as described previously (7). Briefly, 300 to 500 µl of serum were deproteinized by mixing with an equal volume of ice-cold acetonitrile. After 15 min on ice, the samples were centrifuged at 1000 x g for 30 min. The supernatant was buffered with 0.5 ml of 2.5 mm ammonium acetate, pH 9.5, and then applied to a 0.7 x 4-cm column of Affi-Gel 601 (Bio-Rad Laboratories, Richmond, CA) equilibrated with 0.25 mm ammonium acetate, pH 8.5. The nucleosides were eluted with 15 ml of 0.1 M formic acid and then lyophilized. Twenty-five-µl aliquots were injected onto a C18 Bondapak column (Waters Associates, Milford, MA) in a high-performance liquid chromatography system (Waters Associates). The UV chromatographic profiles, at 254 nm, were recorded and integrated by means of a Hewlett-Packard 3385A Laboratory Data System, with 2'-deoxyxguanosine as internal standard. Statistical analysis was done with an Apple II computer (Apple, EUR040K), using Student's t test program.

Transplantable Cells. The AKR tumor line used in this study was developed from a spontaneous thymic lymphoma which arose in our colony (12). It is propagated by the i.v. inoculation of 106 thymocytes into 3- to 4-week-old AKR recipients. The tumor cells infiltrate thymus, lymph nodes, and spleen and form foci in the skin, killing the host at approximately 25 days after inoculation. For assessment of the degree of tumor progression as a function of time (see text), thymuses and spleens were removed from several mice at each time point; they were placed in Bouin's solution for 24 hr and then weighed. The myeloma line

1 This work was supported by the Progetto Finalizzato Controllo Crescita Neoplastica, CNR Rome, Italy.
2 To whom requests for reprints should be addressed.

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has been described in detail elsewhere (4). It is maintained in BALB/c intervals. It grows in disseminated form (spleen and bone marrow) when injected i.p.

Transplantation Test for Leukemic Cells. The entire cell content of the thymus (1 to 2 x 10^6 cells) from each 6-month-old mouse tested was injected i.p. into one AKR recipient, 1 to 2 months old, which was subsequently observed for 3 months for tumor development. Mice were judged to be positive for lymphoma development when palpable lymph nodes were found.

Mitogen Stimulation. Thymocytes from normal and lymphomatous mice were incubated in a microtitre plate in the presence or absence of concanavalin A (10 µg/ml; Sigma Chemical Co., St. Louis, MO). The medium used was an equal mixture of RPMI 1640 (Grand Island Biological Co., Grand Island, NY) and Click's medium (Flow Laboratories, Irvine, Scotland) with 5% heat-inactivated fetal calf serum and 5 x 10^{-5} M 2-mercaptoethanol. After 60 hr, 1 µCi of [3H]thymidine (Radiochemical Centre, Amersham, Buckinghamshire, England; specific activity, 25 Ci/mmol) was added to each well, and incubation was continued for 4 hr, after which the samples were harvested and counted. The cell concentration was 5 x 10^6/ml; the volume was 175 μl.

RESULTS

Levels of Pseudouridine in the Serum of High- and Low-Lymphoma-Incidence Strains of Mice. We determined serum levels of pseudouridine in over 220 AKR (high incidence of lymphoma) and BALB/c (low incidence) mice. The results are given in Chart 1, where the values are grouped according to mouse strain, sex, age (female AKR), and where samples were from lymphomatous mice, according to localized and disseminated disease. Statistical evaluation of the data demonstrated the significance of several trends which can be noted in Chart 1: (a) pseudouridine levels in the serum of young, healthy female AKR mice (up to 5 months of age) are significantly higher than those observed in serum from both male AKR and male BALB/c mice (all ages) (p < 0.01). A sex-related difference was also seen in the BALB/c strain, and the mean pseudouridine concentrations for the same sex were not different between the 2 strains; (b) a significant difference exists between the values obtained with female AKR mice 6 months old and the younger female AKR group (p < 0.001); and (c) pseudouridine levels are significantly higher in AKR mice with clinically advanced disease than in all other groups (p < 0.001).

Increase of Pseudouridine Levels in the Serum of 6-Month-Old Mice (Preneoplastic Period). Because of the difficulty of keeping large numbers of male mice for extended periods of time and since the incidence of lymphoma is lower and the appearance of the disease is somewhat delayed as compared to female mice, we focused on the latter group. In our colony, lymphoma begins to appear after the age of 6 months, and by 8.5 months approximately 50% of the female mice have developed the disease, in agreement with reports for other colonies (10). The group of 6-month-old mice reported in Chart 1 were considered disease free because the thymus was normal in size and appearance and no lymphoma cells were observed in suspensions of thymus examined under the light microscope, lymphoma cells being easily distinguished because of their larger size. It is interesting to note, however, that the pseudouridine levels in these mice ranged from the higher limits for normal to those seen in the serum of mice at an early stage of the disease, where macroscopic changes were observed in the thymus, usually confined to one lobe, at autopsy.

Although the incidence of lymphoma is very low at 6 months and there was no evidence of disease in the 6-month-old mice,

<table>
<thead>
<tr>
<th>Donor</th>
<th>Pseudouridine (nmol/ml)</th>
<th>Pseudouridine development in thymocyte recipient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preneoplastic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>16.88</td>
<td>380 (Donors 1 to 3)</td>
</tr>
<tr>
<td>2</td>
<td>15.44</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10.60</td>
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<tr>
<td>4</td>
<td>10.55</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>11.55</td>
<td>322 (Donors 4 to 6)</td>
</tr>
<tr>
<td>6</td>
<td>7.24</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>13.24</td>
<td>517</td>
</tr>
<tr>
<td>8</td>
<td>9.44</td>
<td>262</td>
</tr>
<tr>
<td>9</td>
<td>18.20</td>
<td>+ (13)</td>
</tr>
<tr>
<td>10</td>
<td>7.90</td>
<td></td>
</tr>
<tr>
<td>Lymphomatous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11(a)</td>
<td>10.56</td>
<td>+ (2)</td>
</tr>
<tr>
<td>12(s)</td>
<td>29.90</td>
<td>8</td>
</tr>
<tr>
<td>13(t)</td>
<td>36.44</td>
<td>1</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>9.47</td>
<td>428</td>
</tr>
<tr>
<td>15</td>
<td>6.33</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>6.90</td>
<td>350 (Donors 15 to 17)</td>
</tr>
<tr>
<td>17</td>
<td>6.60</td>
<td></td>
</tr>
</tbody>
</table>

* Donors 12, 13, and 15 to 17 were male mice; all others were female. Donors 1 to 11 were 6 months old; Donor 12 was 8 months old; Donor 13 was 3 months old; and Donors 14 to 17 were 3 weeks old.

* cpm incorporated by concanavalin A-stimulated culture/cpm incorporated by unstimulated culture. Thymocyte cultures were prepared from individual mice or from pools of 3 donors.

* Numbers in parentheses, time in weeks for lymphoma development after the injection of 1 to 2 x 10^6 thymocytes from the indicated donor mouse into a young, syngeneic recipient. Total observation period, 15 weeks.

* s, mouse with spontaneous lymphoma; t, mouse with T2 transplanted tumor.
we examined the functional activity of thymocytes from these mice to demonstrate the absence of frank neoplastic changes. Thymocytes from young healthy mice, from 6-month-old mice, and from lymphomatous mice were compared for their ability to respond to stimulation with concanavalin A. As shown in Table 1, thymocytes from 6-month-old mice (Donors 1 to 8) and from young healthy mice (Donors 14 to 17) were stimulated to the same extent, whereas little or no stimulation was obtained with an equal number of thymocytes from lymphomatous mice (Donors 13 and 14).

We also transplanted thymocytes from several of the 6-month-old mice indicated in Table 1. Upon autopsy, only one of the mice (Donor 11) was found to have lymphoma; the disease was in an early stage since neoplastic changes were evident only in the thymus. Thymocytes from each of these 6-month-old mice were injected into young syngeneic mice which were then periodically examined for the presence of lymphoma. As shown in Table 1, only the recipient of thymocytes from lymphomatous donor (Donor 11) developed overt disease after a short latency (2 weeks). Of the mice given injections of thymocytes from the apparently disease-free mice (Donors 7 to 10), 3 developed the disease, but only after a long lag period (7 to 13 weeks).

Transplantable AKR Lymphoma in Study of Proportional Increase of Pseudouridine to Tumor Burden. To investigate the relationship between the increase in serum pseudouridine levels and tumor burden, a transplantable AKR lymphoma line (T2) was utilized. In Chart 2, the weight of spleen and thymus and the levels of pseudouridine in the serum are plotted as a function of time after transplantation. The results clearly indicate that the increase of pseudouridine parallels that of tumor burden. In fact, the ratio of pseudouridine level versus tissue weight remains constant (Chart 2, top).

No Increase in Pseudouridine Levels in BALB/c Mice Carrying a Transplantable Myeloma Tumor. Table 2 summarizes the data obtained with a transplantable myeloma tumor. No significant difference was noted between the levels of pseudouridine in the serum of mice carrying MOPC-460 myeloma tumor, either in ascites or disseminated form, and the basal level found for the BALB/c strain. At the time of blood collection, the total number of myeloma cells present in the peritoneal fluid was from 2 to 5 x 10^7 (terminal stage of disease). When the tumor was in disseminated form, the spleen was greatly enlarged (up to 10-

<table>
<thead>
<tr>
<th>No. of mice</th>
<th>Strain</th>
<th>Tumor</th>
<th>Pseudouridine^a^ (nmol/mg)</th>
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</thead>
<tbody>
<tr>
<td>10</td>
<td>BALB/c (female)</td>
<td>MOPC-460 (i.p.)</td>
<td>7.89 ± 3.57^a^</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MOPC-460 (i.v.)</td>
<td>5.90 ± 1.92</td>
</tr>
<tr>
<td>8</td>
<td>BALB/c (male)</td>
<td>None</td>
<td>8.10 ± 2.49^a^</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MOPC-460 (i.v.)</td>
<td>6.69 ± 1.80^a</td>
</tr>
<tr>
<td>5</td>
<td>AKR (male)</td>
<td>T2 (i.p.)</td>
<td>28.67 ± 7.41^a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>6.30 ± 1.50</td>
</tr>
</tbody>
</table>

^a BALB/c mice were 2 to 3 months old at the time of the inoculation with MOPC-460; AKR mice were 1 to 3 months old.
^b Tumors transplanted i.v. grow in disseminated form; i.p. tumors grow in ascites form.
^c Serum samples collected from MOPC-460-bearing mice at 16 to 19 days after inoculation (terminal stage) and from T2 mice at 25 to 30 days.
^d Mean ± S.D.
^e Numbers in parentheses are taken from Chart 1.

fold as judged by weight) and the bone marrow was replaced by tumor cells. Values obtained with serum from mice carrying the AKR lymphoma (T2) are given for comparison.

DISCUSSION
The objective of this investigation was to examine the relationship between pseudouridine serum levels and the development of neoplasia in an animal system. We chose the AKR mouse strain for our studies because of the high predictability of lymphoma development which renders possible the analysis of changes in biological functions related to the transition to a neoplastic state. Our results showed that in the AKR system there is indeed an increase in serum pseudouridine levels with the development of the disease and that the levels are higher in the terminal phase.

The most intriguing finding was the increase of pseudouridine serum levels in 6-month-old female AKR mice prior to the appearance of lymphoma. The mean value obtained for this group was significantly higher than that found in the serum of younger mice of the same colony and very similar to that observed in the early stage of the disease, when the thymus is infiltrated with tumor cells. Several lines of evidence suggested that few, if any, neoplastic cells were present in the thymus of these 6-month-old mice. The observation that lymphoma developed in recipients of thymocytes from these mice only after a long lag period is compatible with an effect of recombinant mink cell focus-inducing virus which is released from thymocytes in the preneoplastic period and which is known to induce an acceleration of lymphoma onset when injected into young syngeneic mice (6). Thus, the increase of pseudouridine in the period just prior to lymphoma development seems to be related to the commitment of thymocytes to undergo neoplastic transformation, as a result of an initial event of lymphomagenesis, and appears to be a precarious biochemical signal of the onset of the disease in AKR mice.

That the increase in pseudouridine level is disease related and not a function of age is supported by the observation that healthy mice of the same age or older, whether syngeneic (male AKR) or from a strain that develops leukemia-lymphoma late in life and with low incidence (male and female BALB/c), did not show any increase of pseudouridine level with age.

The data obtained with transplantable tumors deserve particular comment. Our results with the AKR transplantable lymphoma confirm those found with the spontaneous tumor, since an increase in pseudouridine serum level proportional to tumor burden was observed. This is in good agreement with previous
findings which have shown significant correlation between serum levels and stage of disease in cancer patients (23). On the other hand, in BALB/c mice bearing a transplantable myeloma, a chemically induced tumor of B-lymphocyte origin, pseudouridinoid levels remained within the normal range despite the presence of a large number of tumor cells (Table 2). A similar pattern was seen when the 2 different tumors were, respectively, injected into BALB/c x AKR F1 mice (data not shown); i.e., elevated levels of pseudouridine were found in F1 mice bearing the transplantable AKR lymphoma, but not in F1 mice bearing the BALB/c myeloma. Altogether, these findings restrict the number of hypotheses regarding the mechanisms by which the increase of pseudouridine is generated: (a) it does not appear to be necessarily linked to cell proliferation; (b) not all types of neoplastic cells exhibit abnormal production and release of pseudouridine; and (c) the phenomenon does not appear to be related to a host response to the appearance of neoplastic cells.

One of the hypotheses proposed to explain the elevated levels of modified nucleosides in cancer patients is that the rate of the turnover of tRNA is increased (2). It seems appropriate to extend this hypothesis to the AKR system since it is known that: (a) recombinant viruses are activated in the preneoplastic period; (b) the proline tRNA species which functions as a primer for the reverse transcriptase of murine retroviruses has a higher pseudouridine content (pseudouridine in Loop IV instead of ribothymidine) than do other tRNA species (14); (c) the level of reverse transcriptase-containing particles in the thymus of lymphomatous mice is 20- to 40-fold higher than that present in the thymus of young, healthy mice (11). One may speculate that an increase in the amount and/or in the turnover rate of proline tRNA primer during the preneoplastic period, and after overt lymphoma has occurred, could contribute to the increase of serum pseudouridine; we are now examining this possibility. It is interesting to note that the pseudo sequence is also present in tryptophan tRNA, which serves as the primer of Rous sarcoma virus (15), as well as in arginine tRNA found in mouse leukemia cells (13).

An increase in the level of modified nucleosides, particularly pseudouridine, in the biological fluids of cancer patients has been documented in a large number of studies (3, 5, 9, 17, 21, 23–27, 29). Their use as tumor markers, primarily for monitoring the progression of tumors and their response to treatment, has been proposed; thus far, however, the biological basis of the phenomenon has not been clarified, nor has the specificity or prediction value for cancer diagnosis been studied extensively. Our results offer experimental evidence that in an animal system an increase in serum pseudouridine level precedes the appearance of a neoplastic disease, correlates with tumor burden, and does not seem to be a nonspecific consequence of cell proliferation. If the identity of the phenomenon observed in this murine system and the corresponding human disease can be demonstrated, the system can provide a means for studying the significance of the fluctuations in pseudouridinoid levels observed in cancer patients undergoing treatment and to better assess the utility of this molecule as a tumor marker.

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REFERENCES


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