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Increased Expression of Queuosine Synthesizing Enzyme, tRNA-Guanine Transglycosylase, and Queuosine Levels in tRNA of Leukemic Cells

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Queuosine is a modified nucleoside located at the first position of the tRNA anticodon, which is synthesized by tRNA-guanine transglycosylase (TGT). Although the levels of queuosine in cancer cells have been reported to be lower than those in normal cells, the expression levels of TGT remain to be determined. We determined the expression levels of a subunit of TGT (TGT60KD). Contrary of our expectations, the results revealed higher levels of expression of TGT60KD than that in normal cells, and the level of queuosine in the tRNA fraction corresponded with that of TGT60KD expression. These results suggest the possibilities that the expression levels of TGT60KD regulate TGT activity and the levels of queuosine, and that TGT60KD plays significant roles in carcinogenesis. To our knowledge, this is a first report of increased expression levels of TGT60KD in human cancer cells.

Key words: expression, leukemia, queuosine, tRNA-guanine transglycosylase.

tRNA contains more than eighty kinds of modified nucleosides (*1*, *2*). Modified nucleosides are related to the maintenance of the tRNA structure, and the interaction between codons and anticodons (*3*). We previously reported that increased levels of modified nucleosides, 1-methyladenosine and pseudouridine, were observed in the urine of cancer patients compared with those in normal subjects (*4–6*). The levels of 1-methyladenosine and pseudouridine in urine are particularly increased in patients with leukemia and lymphomas, and the variations in their levels are closely related with the disease status. Borek *et al.* suggested that high turnover rates of RNA in tumor cells caused elevation of modified nucleoside levels in urine (*7*). However, the exact mechanism remains obscure.

Queuosine is a unique modified nucleoside (*8*, *9*). It is located only at the first position of the anticodon of tRNA accepting asparagine, aspartic acid, tyrosine, and histidine. Queuosine is synthesized through a base-exchange reaction between guanosine in tRNA and queuine, a base moiety of queuosine (*10*, *11*). This reaction is catalyzed by tRNA-guanine transglycosylase (TGT). Recently, a 60 kDa subunit (TGT60KD) of TGT was cloned by Desphande *et al.* (*12*). However, since the recombinant TGT60KD protein *per se* did not exhibit queuosine synthesis activity, the presence of other subunit(s) is assumed to be necessary for the activity.

Queuosine has been associated with cancer. Decreased levels of queuosine are observed in cancer tissues compared with in normal tissues (*13–16*). On the other hand, protein kinase C, which is one of the key enzymes involved in cell transformation, is reported to activate TGT, resulting in increased levels of queuosine in tRNA (*17*, *18*). Recently, Morris *et al.* showed the possibility that a queuosine deficiency in cancer cells can occur for three reasons: (i) a deficiency of uptake of queuine into cells, (ii) a deficiency in the incorporation of queuine into tRNA by TGT, and (iii) a deficiency in queuosine salvage at tRNA degradation (*19*). They demonstrated that the last two reasons explain the queuosine deficiency in cultured cancer cell lines.

In this study, we observed the levels of the TGT60KD protein in leukemic cells. Contrary to our expectations, the expression levels of the TGT60KD protein in leukemic cells were increased compared with those in normal cells. Furthermore, the queuosine levels in the tRNA fraction of leukemic cells were also increased. To our knowledge, this is the first report regarding increased expression levels of TGT60KD in human cells.

MATERIALS AND METHODS

Cell Culture—Human leukemic cell lines, Jurkat, PEER, HUT-78, HPB-ALL, and Molt-4F, were obtained from the Cell Resource Center for Biomedical Research, Tohoku University. Other leukemic cells were kindly provided by Dr. Aso of Tohoku University. BALB/3T3, a mouse fibroblast-like cell line, and SV-T2, a SV40 virus-transformed cell line of BALB/3T3, were from the Japanese Cancer Research Bank. Leukemic cells were grown in RPMI 1640 medium containing 10% heat-inactivated calf serum, 0.2% glucose, 1 mM pyruvate, 2 mM glutamine, 100 units/ml penicillin,

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Abbreviations: AML, acute myelogenous leukemia; CLL, chronic lymphocytic leukemia; GST, glutathione-S-transferase; HRP, horseradish peroxidase; PBMC, peripheral blood mononuclear cells; TGT, tRNA-guanine transglycosylase; TGT60KD, a subunit of TGT; UBP, ubiquitin-specific processing protease.

100 μg/ml streptomycin, and 60 μg/ml kanamycin at 37°C under a humidified atmosphere of 5% CO_2. Fibroblast cells were cultured in MEM medium with the additives mentioned above.

Preparation of the Antibody for TGT60KD—Recombinant TGT60KD was prepared as a fusion protein of glutathione-S-transferase (GST). TGT60KD cDNA (GenBank Accesion No. U30888) was amplified by RT-PCR (RNA PCR kit; Takara, Shiga). Total RNA purified from K562 and a pair of primers (60K-F, 5′-GCGGATCCATGCCGCTCTACTCCG-3′; 60K-R, 5′-CTAAGCATGAATTCTAAAATGAAGATTA-3′) were used as the template and primers, respectively. PCR was performed, *i.e.* 30 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 2 min. Amplified TGT60KD cDNA was cloned into the pGEX-2T vector (Pharmacia), to prepare the GST-TGT60KD fusion protein and the pRSET-A vector (Invitrogen), for the histidine-tagged TGT60KD fusion protein. The GST-TGT60KD and histidine-tagged TGT60KD fusion proteins were purified using the standard protocol. BALB/c mice were subcutaneously and intraperitoneally hyperimmunized with the GST-TGT60KD fusion protein, and the prepared hybridomas secreting the antibody specific for TGT60KD were screened using the histidine-tagged TGT60KD fusion protein. The monoclonal anti-body specific for TGT60KD was selected.

Immunoblotting—Cells were lysed in a lysis buffer containing 20 mM Tris (pH 7.4), 0.1% SDS, 1% Triton X, and 100 μM Pefabloc SC (Merck), followed by three cycles of freezing and thawing. The lysate was centrifuged (900 ×*g*, 20 min, 4°C) and the resultant supernatant was used as a sample. Samples were separated by 8% SDS-PAGE and then blotted onto a nitrocellulose membrane. The membrane was incubated with 10 μg/ml of the anti-TGT60KD monoclonal antibody, followed by incubation with rabbit anti–mouse IgG (Zymed) and horseradish peroxidase (HRP)–Protein G (Zymed). Diaminobenzidine was used as the substrate for HRP. All procedures were performed at room temperature. The densities of the bands were determined with NIH image software. All cells from the normal subjects and patients with leukemia in this study were collected with their informed consent. Peripheral blood mononuclear cells (PBMC) were purified using Histopaque 1077 (Sigma) according to the manufacturer's instructions.

Preparation of the tRNA Fraction—Purification of the tRNA fraction from the cells was carried out by the method of Morgan *et al.* (*20*). Total RNA of the cells was extracted using Isogen (Nippon Gene) according to the manufacturer's instructions. The obtained total RNA was mixed with a 0.1 volume of 20% potassium acetate (pH 4.5) and a 0.2 volume of 12 M LiCl, followed by incubation on ice for 4 h to precipitate high-molecular-weight RNA. After centrifugation, 3 volumes of ethanol was added to the supernatant, followed by incubation overnight at 4°C. The tRNA fraction was obtained as a pellet on centrifugation. Yeast tRNA fraction was purified by the method of Holley *et al.* (*21*).

Determination of Queuosine Deficiency in the tRNA Fraction—Recombinant *Escherichia coli* TGT was prepared as a fusion protein of GST. The *E. coli* TGT sequence (GenBank Accesion No. M63939) was amplified by PCR (RNA PCR kit, Takara). DNA extracted from *E. coli* K12 and a pair of primers (E-TGT-F, 5′-GGGGATCCATGAAATTTGAACT-3′; E-TGT-R, 5′-CCCTGAATTCTAATATTAATCA-3′) were used as the template and primers, respectively. PCR was performed, *i.e.* 30 cycles of 72°C for 2 min, 91°C for 1 min, and 45°C for 1 min. The amplified *E. coli* TGT sequence was cloned into the pGEX-2T vector (Pharmacia) to prepare the GST–*E. coli* TGT fusion protein. The GST–*E. coli* TGT fusion protein was purified using the standard protocol. GST was not removed from the GST–*E. coli* TGT fusion protein, since this fusion protein exhibited TGT activity and its activity was unstable on further treatment.

The queuosine deficiency in tRNA was determined by the method of Baranowski *et al.* with slight modification (*14*). In brief, 100 μl of the reaction mixture, containing 100 mM HEPES (pH 7.5), 20 mM $MgCl_2$, 100 nCi of [^{14}C]guanine, 1.5 μg of GST–*E. coli* TGT and the extracted tRNA fraction, was incubated at 37°C for 2 h. After the addition of 200 μl of ethanol, the tRNA fraction was recovered on a membrane filter (Kurabou), and then its radioactivity was counted with a liquid scintillation counter. The obtained values were standardized and expressed as ratios with respect to the yeast tRNA equivalent in a simultaneous experiment, since yeast tRNA does not contain queuosine (*13*).

Determination of 1-Methyladenosine and Pseudouridine in Urine—The levels of 1-methyladenosine and pseudouridine in urine were determined by the method of Itoh *et al.* (*5*, *6*).

RESULTS

Expression of TGT60KD in Leukemic Cell Lines—To determine the expression levels of TGT60KD in leukemic cells, an antibody for TGT60KD was prepared. The expression levels of TGT60KD in PBMC from normal subjects and myelogenous leukemic cell lines were determined by immunoblotting using the anti-TGT60KD monoclonal antibody (Fig. 1, A and B). The expression of TGT60KD was detected in all cells including PBMC from normal subjects. The expression levels of TGT60KD were increased in HEL, K562, and KG-1, being approximately 2.0-, 4.2-, and 3.8-fold higher than that in normal PBMC, respectively. The expression levels of TGT60KD in HL-60 and THP-1 were comparable to that in normal PBMC. We also determined the expression levels of TGT60KD in lymphocytic leukemic cell lines (Fig. 1C). The expression levels of TGT60KD in HPB-ALL, Jurkat, and Molt-4F were approximately 3.0-, 2.5-, and 2.0-fold higher than in PBMC of normal subjects, respectively. The expression levels of TGT60KD in PEER and HUT78 were comparable to that in PBMC.

Queuosine Deficiency in the tRNA Fraction of Lymphocytic Leukemic Cell Lines—The levels of queuosine deficiency in the tRNA fraction were determined by means of a guanine incorporation assay. In the assay system, an increased level of guanine incorporation into the tRNA fraction implies a decreased level of queuosine in the tRNA fraction. To examine the reliability of the assay, we determined the levels of queuosine deficiency in BALB/3T3 and SV-T2, virus-transformed cells of BALB/3T3. Katze has shown that the levels of queuosine deficiency of tRNA accepting asparagine in SV-T2 cells are higher than that in BALB/3T3 (*22*). Consistent with this report, our results for the tRNA fraction revealed that the levels of queuosine deficiency in SV-T2 were twice higher than that in BALB/3T3 (data not shown). Therefore, we regarded the assay as being reliable enough and thus used it for further experi-

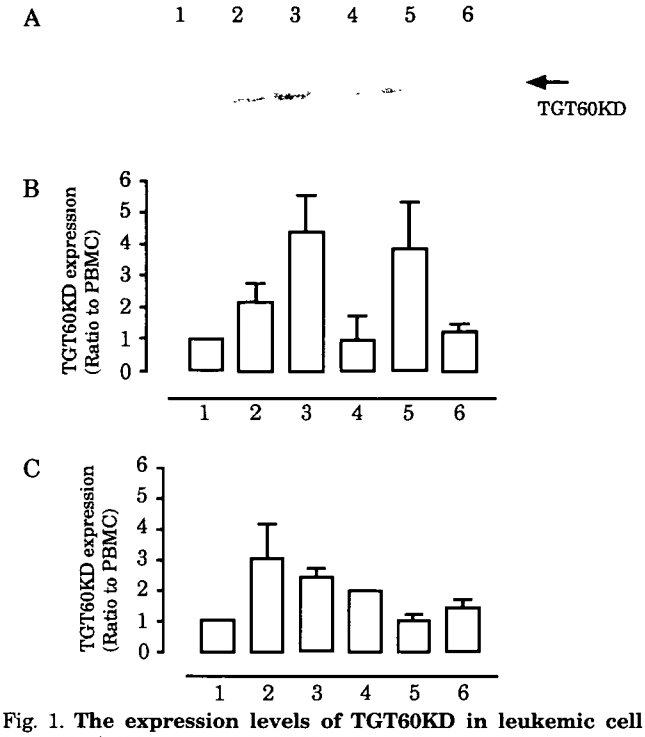

Fig. 1. **The expression levels of TGT60KD in leukemic cell lines.** A: Cell lysates (50 μg) of human myelogenous leukemic cell lines were electrophoresed in 8% SDS–polyacrylamide gels, transferred to a nitrocellulose membrane, and then reacted with the anti TGT60KD antibody (1, PBMC; 2, HEL; 3, K562; 4, HL-60; 5, KG-1; 6, THP-1). B: The intensities of the visualized bands on immunoblotting of myelogenous leukemic cell lines were calculated using NIH image software and are shown as the ratio to the average intensity of PBMC (1, PBMC; 2, HEL; 3, K562; 4, HL-60; 5, KG-1; 6, THP-1). C: The intensities of the visualized bands on immunoblotting of lymphocytic leukemic cell lines were calculated using NIH image software and are shown as the ratio to the average intensity of PBMC (1, PBMC; 2, HPB-ALL; 3, Jurkat; 4, Molt-4F; 5, PEER; 6, HUT78).

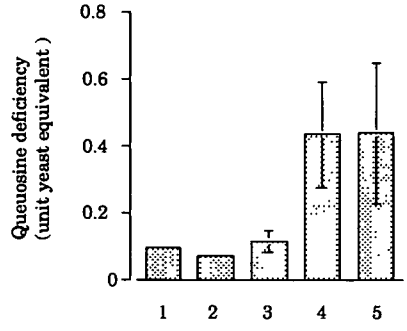

Fig. 2. **Queuosine deficiency levels in the tRNA fraction of human lymphocytic leukemic cell lines.** The tRNA fraction was extracted and the levels of queuosine deficiency were determined as the [^{14}C]guanine incorporation into tRNA. The values are shown as ratios with respect to the yeast tRNA equivalent (1, HPB-ALL; 2, Jurkat; 3, Molt-4F; 4, PEER; 5, HUT78).

TABLE I. **Determination of the levels of queuosine deficiency in peripheral blood mononuclear cells, and those of 1-methyladenosine and pseudouridine in urine.** The tRNA fraction of PBMC was extracted and levels of queuosine deficiency were assayed as the [^{14}C]guanine incorporation into the tRNA fraction. The values are shown as ratios with respect to the yeast tRNA equivalent. The 1-methyladenosine and pseudouridine levels in urine were determined as previously described (*5*, *6*). The normal subject and cut-off values were cited from previous reports (*5*, *6*).

	Queuosine deficiency (unit yeast equivalent)	1-Methyladenosine (nmol/μmol creatinine)	Pseudouridine (nmol/μmol creatinine)
CLL[a]	0.0370	3.00	68.5
AML[b]	0.0433	2.94	67.0
AML	0.0685	3.02	78.9
AML	0.0846	2.21	71.8
Mean	0.0584±0.0222	—	—
Normal subject	0.1218±0.0064	1.91	31.2
Cut-off value	—	3.23	51.0

[a]CLL, chronic lymphocytic leukemia; [b]AML, acute myelogenous leukemia.

ments. The levels of queuosine deficiency in HPB-ALL, Jurkat, and Molt-4F cells were lower than those in PEER and HUT78 (Fig. 2). Therefore, the levels of queuosine in the tRNA fraction in HPB-ALL, Jurkat, and Molt-4F cells were higher than those in PEER and HUT78. The levels of queuosine correlated with those of TGT60KD expression in these cell lines (Fig. 1C).

Queuosine Deficiency in the tRNA Fraction of PBMC of Patients with Leukemia—The levels of queuosine deficiency in the tRNA fraction of PBMC of patients with leukemia were determined by means of the guanine incorporation assay. The queuosine deficiency in PBMC of one patient with chronic lymphocytic leukemia (CLL) and three patients with acute myelogenous leukemia (AML) was attenuated compared with that in normal subjects (Table I). PBMC of patients with leukemia contain higher levels of queuosine than those of normal subjects. The levels of modified nucleosides, 1-methyladenosine and pseudouridine, in the urine of the patients with leukemia were also more increased than those in normal subjects. The levels of pseudouridine in the urine of all the four patients exceeded the cut-off value.

DISCUSSION

The expression levels of TGT60KD have not been studied to date, although TGT activities have been examined in many cells (*19*, *23*). To our knowledge, this is the first study on the expression levels of TGT60KD. Our results reveal that the expression levels of TGT60KD increased in both myelogenous and lymphocytic leukemic cell lines, which suggests that increased expression of TGT60KD is a characteristic of leukemic cells irrespective of the cell type. Emmerich *et al.* reported decreased levels of queuosine in human leukemia and lymphoma cells compared with those in nonneoplastic tissues (*24*). Although there appear to be discrepancies between their report and ours, some reasons should be considered. The first is that the expression levels of TGT60KD are not correlated with the levels of queuosine. The queuosine levels can be influenced by several factors; the uptake of queuine into cells across the plasma membrane, the salvage of queosine in cells, and TGT activity modulated by protein kinase C activity (*19*). However, the contributions of these factors seem relatively low because our results show that the expression levels of TGT-60KD closely correspond to the queuosine levels in lympho-

cytic leukemic cell lines. The second reason is the difference in the samples used. Emmerich *et al.* used cells from tonsils, lymph nodes, and spleens with inflammation as controls, in which there are up to tenfold differences in the queuosine deficiency (*24*). We used PBMC from normal subjects as controls. Shindo-Okada *et al.* reported that the levels of queuosine decreased as murine erythroleukemic cells differentiated (*25*). By contrast, Parniak *et al.* reported that the levels of queuosine increased during differentiation (*26*). Since there is little information on the effects of inflammation, tissue differences and differentiation on queuosine deficiencies, and information on the expression levels of TGT60KD in cells is also limited, more information needs to be collected. In addition, it has been reported that a protein of 80 kDa with TGT activity was purified from rat liver, although the distribution of the protein remains obscure (*27*). It is possible that the protein influences the levels of queuosine, at least in the liver.

Although our results showed the increased expression levels of TGT60KD in leukemic cells, the biological role of TGT60KD remains obscure. We have no direct evidence with respect to the relationship between increased levels of TGT60KD expression and development of cancer. TGT-60KD exhibits sequence homology with the ubiquitin-specific processing protease (UBP) family, and the two characteristic sequences of the UBP family, the Cys and His boxes, are conserved in its sequence (*8*). UBP is a thiol protease specific for the cleavage of the peptide bond at the carboxyl terminal Gly of ubiquitin (*28*). Ubiquitination and deubiquitination systems are related to cell proliferation through protein degradation, since cell-cycle-regulating proteins such as p53 are degraded by these systems (*29*). One of the members of the UBP family, tre-2, has also been reported to be an oncogene (*30*). It is possible that TGT-60KD also exhibits UBP-like activity and influences ubiquitin-dependent protein degradation, accelerating the cell proliferation of cancer cells.

We have reported that patients with cancer, particularly leukemia, and lymphomas, excrete increased amounts of pseudouridine and 1-methyladenosine in the urine compared with normal subjects (*4–6*). The results obtained in this study correspond with our previous report. Borek *et al.* reported that increased excretion levels of modified nucleosides in cancer patients resulted from a high turnover rate of RNA in tumor cells (*7*). Here, we observed that the level of queuosine in PBMC is also increased in patients with leukemia who excrete increased levels of pseudouridine in their urine. This implies that in leukemic cells at least some RNA species are extraordinarily modified irrespective of the RNA turnover rate, since the levels of queuosine shown here were standardized against the levels of RNA. Understanding of the RNA modification mechanism in cancer cells could provide a clue as to the comprehension of cancer biology and development of new therapies.

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