Processing Enzymes Acting on Carbohydrate Moiety of Lysosomal Hydrolases in Leukemic Cells: Elevated Activity of N-Acetylglucosamine-1-Phosphotransferase

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We previously demonstrated that an acidic variant form of lysosomal arylsulfatase B accumulated in chronic myelogenous leukemia (CML) cells was highly phosphorylated at its carbohydrate moiety (Uehara Y. et al. Cancer Res 43:5618, 1983). Since lysosomal hydrolases including the sulfatase underwent the posttranslational phosphorylation processing at the carbohydrate moiety, we investigated two enzymes acting on the processing in peripheral leukocytes from leukemia patients. The activity level of the first enzyme in the processing, an N-acetylglucosamine-1-phosphotransferase to form phosphodiester at the carbohydrate moiety, was significantly higher in CML cells than in normal control. The transferase level in CML cells was also higher compared with that in normal bone marrow cells, which include myeloid progenitor cells. However, the activity of the second processing enzyme, a phosphodiester glycosidase that converts a phosphodiester to a phosphomonoester, showed no consistent change in CML cells. Thus, increment of the sulfatase variant containing phosphomonoesters and diesters in CML cells is most probably associated with elevated activities of the phosphotransferase. In two cases of CML in blastic crisis and a case of acute myelogenous leukemia (AML), activity of the processing enzyme was considerably decreased concomitant with reduction of peripheral blastic cells by chemotherapy.

ACTIVITY LEVELS of many lysosomal hydrolases increase in human cancer tissues and leukemic cells compared to those in the normal control. In addition, malignant tissues and cells were often accompanied by negatively charged variant forms of the hydrolases.1 Lysosomal arylsulfatase B from human chronic myelogenous leukemia (CML) cells showed a considerable increase in the amount of the acidic variant form, with the isoelectric point (pI) of about 7.5 as compared with the hydrolase with pI 8-9 in leukocytes from healthy subjects.2,3 Since most of all the lysosomal hydrolases were glycoproteins by nature,2 formation of the acidic variants was examined for modification on the protein and carbohydrate moieties of the hydrolases. As to arylsulfatase B, acidic variants in CML cells were demonstrated to be heavily phosphorylated not only on the carbohydrate chains,2 but also on the protein3 as in the observations on lung cancer.4,5

Phosphorylation on the carbohydrates of the CML sulfatase involved phosphomonoester and phosphodiester, which was largely responsible for the appearance of the acidic variants in CML cells.2 However, the mechanism by which phosphorylation on the carbohydrate chains bound to the sulfatase is increased in CML cells has yet to be elucidated. High mannose-type carbohydrate chains of lysosomal matrix hydrolases undergo a series of posttranslational processing for targeting to lysosomes.4,5 The processing involves acquisition of phosphomannosyl residue, which is a recognition marker for intracellular transport of the hydrolases. This recognition marker is generated by the sequential action of two enzymes in Golgi apparatus. First, UDP-N-acetylglucosamine-lyosomal enzyme N-acetylglucosamine-1-phosphotransferase (GlcNAc-phosphotransferase) transfers N-acetylglucosamine-1-phosphate exclusively to mannose residues of high mannose-type oligosaccharides on lysosomal hydrolases to give rise to a phosphodiester intermediate. Then, N-acetylglucosamine phosphodiester α-N-acetylglucosaminidase (phosphodiester glycosidase) removes the N-acetylglucosamine residues to expose the phosphomonoester recognition signal.6

In this study we have investigated whether changes in the activity levels of these two processing enzymes for lysosomal hydrolases are responsible for increased generation of phosphorylated oligosaccharides on arylsulfatase B in leukemic cells compared to normal leukocytes.

MATERIALS AND METHODS

Reagents. UDP-N-acetylglucosamine, ATP, N-acetylgalactosamine, N-acetylmannosamine, α-methylmannoside, QAE-Sephadex (Q-25-120) were from Sigma, St Louis. UDP-[6-3H]N-acetylglucosamine (15 Ci/mmol) was from New England Nuclear, Boston. [α-32P]UDP-GlcNAc (1.2 mCi/mmol) were prepared by the method of Reitman and Kornfeld.5 Monoclonal antibody J5 was purchased from Ortho Pharmaceutical, Raritan, NJ. Other chemicals were of analytical grade obtained from various sources.

Patients. Leukemic cells from heparinized peripheral blood were obtained from 23 patients with various types of leukemia. All leukocytes were obtained from adult patients: 13 patients with CML in chronic phase, four patients with CML in blastic crisis, four patients with acute myelogenous leukemia (AML) (M1 and M2 according to French, American, British Nomenclature Committee8), and three patients with acute lymphoblastic leukemia (ALL) (L2). Cytogenetic examinations on peripheral blood and bone marrow cells demonstrated the presence of Ph1 chromosome in all patients with CML.

Cells. Heparinized fresh blood from healthy volunteers and patients with leukemia was subjected to dextran sedimentation to fractionate leukocytes. Leukocytes from healthy volunteers were separated into granulocytes and mononuclear cells by centrifugation with Ficol-Hypaque gradient. In granulocyte fraction contaminating RBCs were removed by hypotonic lysis.

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Normal myeloid cells from bone marrow of healthy volunteers, which include myeloid precursors, were obtained by centrifugation with Ficoll-Hypaque gradient.

**Assay of the GlcNAc-phosphotransferase.** GlcNAc-phosphotransferase activity was assayed according to the method of Reitman and Kornfeld using α-methylmannoside as an acceptor. The reaction mixture contained [β-32P]UDP-GlcNAc (500,000 cpm), 200 mmol/L α-methylmannoside, 5 mmol/L ATP, 50 mmol/L N-acetylglucosamine, 100 μmol bovine serum albumin, 10 mmol/L sodium molydate, 0.25 mmol/L dithiothreitol, 0 mmol/L L-MgCl₂, 10 mmol/L MnCl₂, 1% Lubrol, 100 to 300 μg of protein from cell homogenates and 50 mmol/L Tris-HCl buffer, pH 7.4, in a final volume of 50 μL. Following incubation for 60 minutes at 37°C, the reaction was terminated by the addition of 1 mL of 5 mmol/L sodium EDTA, pH 7.8. The reaction mixture was applied to a QAE-Sephadex column (1.0 x 2.2 cm) previously equilibrated by washing it with 2 mL of buffer A, the product, [32P]GlcNAc-P-α-methylmannoside was eluted with 4 mL of 30 mmol/L NaCl in buffer A and assayed for radioactivity in 12 mL of a scintillation mixture. One unit of phosphodiester glycosidase was defined as 1 pmol of N-acetylglucosamine released per hour.

**Assay of the lysosomal hydrolases.** Activities of arylsulfatases A and B were separately assayed in leukocytes homogenates using p-nitrocatechol sulfate as a common substrate by the method of Baum et al. with a slight modification. One unit of arylsulfatase is defined as the amount of 1 mmol of p-nitrocatechol liberated per hour. Cathepsin D activity was assayed as reported previously.

**RESULTS**

Activity level of GlcNAc-phosphotransferase in leukemic cells. Km value of GlcNAc-phosphotransferase for α-methylmannoside in normal and CML cells was 60 to 80 mmol/L, which was lower than that (158 mmol/L) in the rat liver enzyme. Tables 1 and 2 show activities of leukemic GlcNAc-phosphotransferase from individual patients with...
various leukemias together with their differential counts of peripheral leukocytes and clinical stages. The transferase activity encompassed a wide range between leukemia classes, and even in a single class. The transferase activity in CML in chronic phase was not correlated with the total and differential counts of leukocytes (Table 1). Figures 1 and 2 show individual variations of GlcNAc-phosphotransferase within established diagnostic groups in terms of protein concentration and cell number, respectively. When the transferase level was compared at clinical stages, the activity level in CML cells in chronic phase (mean ± SE, 72.0 ± 8.6 U/mg protein, n = 16, or 143.6 ± 37.1 U/10⁶ cells, n = 12) was significantly (P < .01) higher than that in normal granulocytes (39.0 ± 6.5 U/mg protein, n = 10, or 24.0 ± 4.0 U/10⁷ cells, n = 10). Normal myeloid cells from bone marrow showed similar activity (42 and 50 U/mg protein, n = 2) with that of normal peripheral cells. On the other hand, although two patients (cases no. 12 and 15) in blastic crisis showed an elevated GlcNAc-phosphotransferase level as compared with CML patients in chronic phase, the activity level in four patients encompassed a wide range (279.9 ± 189.2 U/mg protein, n = 4, or 163.2 ± 98.9 U/10⁷ cells, n = 4). The transferase activity in terms of cell number of patients no. 12, 15, and 16 whose blasts were predominantly of the peroxidase-positive myeloid phenotype was higher than that of case no. 14 who had a mixed cell crisis including peroxidase-positive myeloid cells and monoclonal antibody J5-positive lymphoid cells (Table 2). On the other hand, AML cells that consisted of 84% (case no. 19, M1), 54% (case no. 18, M2) and 89% (case no. 17, M1) myeloblasts showed an increase in the activity of transferase, as compared with the cell populations with lower myeloblasts (35%, case no. 20 who was transformed from myelodysplastic syndrome) or with normal granulocytes, although the number of cases is not large enough (Table 3).

In three patients the activity of ALL cells in lymphoid leukemias (Fig 1), remained unchanged and was lower than the range in the normal lymphocytes (120.9 ± 12.1 U/mg protein, n = 9, or 46.1 ± 7.7 U/10⁷ cells, n = 9). In normal leukocytes the activity of the transferase was significantly (P < .01) higher in lymphocytes than in granulocytes. Increased activity of the transferase, thus, is most likely to be confined to leukemic cells of not lymphoid but myelogenous origin.

Activity level of phosphodiester glycosidase in leukemic cells. Phosphodiester glycosidase activity in CML cells of four cases out of eight was higher than either those in normal granulocytes (Table 1 and Fig 3) or normal bone marrow cells (0.1 and 0.09 U/mg protein), implying that the increased activity could not be consistent. The activity of CML cells in blastic crisis was elevated as compared with that in chronic phase (Table 2, Fig 3). When AML cells were assayed for the glycosidase activity, the activity showed higher levels as compared with that in normal granulocytes although number of cases is not large. In normal leukocytes, the phosphodiester glycosidase activity level of lymphocytes (0.43 ± 0.043 U/mg protein, n = 4) was higher (P < .01) than that in granulocytes.
Activities of lysosomal hydrolases and possible relation with processing enzyme level in leukemic cells. In order to examine whether the activity level of GlcNAc-phosphotransferase correlates with the levels of lysosomal hydrolases that contain high mannose-type oligosaccharides, three lysosomal enzymes, namely arylsulfatase A, arylsulfatase B, and cathepsin D, were assayed for the activities (Fig. 4). The activity levels of two arylsulfatases (for arylsulfatase A, 92.0 ± 9.9 U/mg protein, n = 12; for arylsulfatase B 163.5 ± 20.2 U/mg protein, n = 12) in CML cells were higher than those (for arylsulfatase A, 64.8 ± 3.8 U/mg protein, n = 6; arylsulfatase B 129.1 ± 21.5 U/mg protein, n = 7) in normal granulocytes. However, elevation of the activity levels of processing enzymes did not closely correlate with the activities of three hydrolases in CML cells in chronic phase. In normal leukocytes, the levels of two arylsulfatase activities in lymphocytes were similar to those in granulocytes. The activity of cathepsin D (0.021 ± 0.0038 U/mg protein, n = 9) in CML cells also increased compared with that (0.0054 ± 0.0003 U/mg protein, n = 4) in normal granulocytes.

Changes in activity levels of processing enzymes after chemotherapy. Both the GlcNAc-phosphotransferase and phosphodiester glycosidase activities, which were higher in pretreatment state than those of normal granulocytes, were normalized after chemotherapy in the cells from patients with CML. The activity of a processing enzyme (case no. 12) was slightly higher in chronic phase than that in normal granulocytes (Table 1). However, activities of two processing enzymes were markedly elevated in blastic crisis (case no. 12.1) and decreased (case no. 12.2) concomitant with reduction of blast cells after chemotherapy with vincristine, 6-mercaptopurine, and prednisolone (Table 2). Similarly, in case no. 16, the GlcNAc-phosphotransferase activity in blastic crisis decreased in accordance with the reduction of blast cells (case no. 16.1).

In a patient with AML (cases no. 17 and 17.1) (Table 3), the enzyme activities were also normalized concomitantly with a decrease in the number of leukemic cell on complete remission by induction chemotherapy with daunorubicine, cytosine arabinoside, and prednisolone.

DISCUSSION

It is well-known that the processing enzymes acting on carbohydrate moiety of lysosomal hydrolases play a major role in transport into lysosomes in fibroblasts. However, few studies have been made on the processing enzymes in malignant cells.

Lysosomal arylsulfatase B enzyme of CML cells and normal leukocytes was separated into the basic B enzyme and its anionic variant form (B, enzyme) phosphorylated at carbohydrate moiety, on anion-exchange chromatography. The amount of arylsulfatase B, relative to total arylsulfatase B (B + B,) was considerably increased in CML cells compared with normal leukocytes. The increment of arylsulfatase B in CML cells was demonstrated to be due to increased phosphorylation at carbohydrate moiety. The highly phosphorylated sulfatase included both the phosphomonoester- and diesterforms.

In the present study, the activity of GlcNAc-phosphotransferase, which phosphorylates carbohydrates of lysosomal hydrolases, increased in CML cells compared with that in normal granulocytes. On the other hand, the activity level of phosphodiester glycosidase did not show consistent change in CML cells. Transformation to myeloid crisis from chronic phase in some patients with CML brought about more elevation of the activity of the transferase. On the other hand, the processing enzyme activity was decreased in parallel with reduction of peripheral blasts in the course of chemotherapy in patients with CML in blastic crisis and AML. The relative amounts of arylsulfatase B, of CML cells in chronic phase were also decreased concomitantly with an increase in the less phosphorylated form of arylsulfatase B on

Fig 2. Activity level of GlcNAc-phosphotransferase in normal leukocytes and leukemic cells on the basis of cell number (units/10⁷ cells). Bars indicate mean ± SE, P < .01, normal lymphocytes v normal granulocytes, and CML cells v normal granulocytes.

Fig 3. Activity of phosphodiester glycosidase in normal leukocytes and leukemic cells on the basis of protein amount (units/mg protein).
Fig 4. Activity levels of lysosomal hydrolases in normal leukocytes and leukemic cells (units/mg protein). A. arylsulfatase A; B. arylsulfatase B; C. cathepsin D.

Chemotherapy. These results suggest that elevation of the transferase activity is responsible for increase of immature, heavily phosphorylated hydrolases in CML cells. Since the transferase activity in normal bone marrow myeloid cells that include precursor cells was in similar level with that of normal peripheral granulocytes, elevation of the transferase is closely associated with a property of malignancy rather than a phenotype of immature myeloid cells.

An inherited lysosomal storage disease, I-cell disease (mucolipidosis type II) has been characterized by being considerably decreased in lysosomal hydrolases in cultured skin fibroblasts due to profound deficiency of GIeNAc-phosphotransferase. However, the liver spleen, kidney, brain, and leukocytes from the patients had almost normal levels of the lysosomal hydrolases in spite of the defective transferase activity in these sources. These observations led to the suggestion that, in cells other than skin fibroblasts, there may be a targeting mechanism distinct from the mannose-6-phosphate recognition marker on fibroblast hydrolases. Whether the increment in heavily phosphorylated lysosomal sulfatase, which is ascribed to elevated processing enzyme activities, has an effect on lysosomal enzyme targeting events is unclear. Thus, the pathological significance of the increment of phosphorylated hydrolase intermediate forms due to elevation of the processing enzymes in leukemic cells remains to be solved.

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Processing enzymes acting on carbohydrate moiety of lysosomal hydrolases in leukemic cells: elevated activity of N-acetylglucosamine-1-phosphotransferase

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