Human leukocyte glycosylasparaginase: cell-to-cell transfer and properties in correction of aspartylglycosaminuria

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Abstract Aspartylglycosaminuria (AGU), a severe lysosomal storage disease, is caused by the deficiency of the lysosomal enzyme, glycosylasparaginase (GA), and accumulation of aspartylglucosamine (GlcNAc-Asn) in tissues. Here we show that human leukocyte glycosylasparaginase can correct the metabolic defect in Epstein–Barr virus (EBV)-transformed AGU lymphocytes rapidly and effectively by mannose-6-phosphate receptor-mediated endocytosis or by contact-mediated cell-to-cell transfer from normal EBV-transformed lymphocytes, and that 2–7% of normal activity is sufficient to correct the GlcNAc-Asn metabolism in the cells. Cell-to-cell contact is obligatory for the transfer of GA since normal transformed lymphocytes do not excrete GA into extracellular medium. The combined evidence indicates that cell-to-cell transfer of GA plays a main role in enzyme replacement therapy of AGU by normal lymphocytes.

Key words: Aspartylglucosylaminase; Bone marrow transplantation; Cell-to-cell contact; Endocytosis; Lysosomal storage disease

1. Introduction

Aspartylglycosaminuria (AGU, McKusick 208400) is the most common disorder of glycoprotein degradation [1]. It is caused by a deficient activity of the lysosomal enzyme glycosylasparaginase (GA) (glycoasparaginase, aspartylglycosylaminase, aspartylglucosaminidase, EC 3.5.1.26). GA is transported through a mannose-6-phosphate receptor-mediated pathway from the endoplasmic reticulum or extracellular sites into lysosomes, where it participates in the glycoprotein catabolism by cleaving the *N*-glycosidic linkage between *N*-acetylglucosamine and L-asparagine [2]. Deficiency of GA activity in AGU cells leads to the accumulation of aspartylglucosamine (GlcNAc-Asn) and other glycoasparagines in body fluids and tissues of AGU patients [3]. This recessively inherited multisystem disease is characterized by progressive psychomotor retardation, and structural abnormalities in the connective tissue and skeleton [4].

Certain lysosomal enzymes are transferred from normal lymphocytes to fibroblasts from patients with lysosomal storage diseases [5]. Lymphocytes can play an important role in the transfer of corrective enzymes into cells throughout the body in the treatment of lysosomal diseases by bone marrow transplantation [6]. As a result of the presence of the donor enzyme, clearance of the accumulating substrate, and repair of damage and prevention of degenerative disease in the organs affected by the disease can be shown [7]. The transfer of lysosomal enzymes can occur by two distinct mechanisms. One is active internalization of the enzymes into lysosomes via mannose-6-phosphate receptor-mediated endocytosis [8]. A second, quite distinct process involves the transfer of certain lysosomal enzymes from lymphocytes to enzyme-deficient cells by cell-to-cell contact [5,7]. The efficacy of bone marrow transplantation to prevent and reverse the disease process varies from one lysosomal disorder to another [7], suggesting that complex and disease-specific factors in transfer of different lysosomal enzymes are involved. These include various cellular or enzyme-related factors such as poor excretion of enzymes out of normal lymphocytes, rapid degradation of the enzyme protein during the transfer process, ineffective transport of the enzyme into lysosomes of the recipient cells, rapid degradation of the correcting enzyme in the target cells or the requirement for a high intracellular enzyme activity to correct the turnover of the lysosomal substrate.

Bone marrow transplantation has beneficial effects on the natural cause of AGU [9], but the factors that are involved in the transfer of GA from normal to AGU cells and the efficiency of this process in correcting the metabolism in the diseased cells are not fully understood. We have now examined the transfer of human leukocyte GA from extracellular medium and cell-to-cell from normal lymphocytes to AGU lymphocytes and fibroblasts in order to understand the disease-specific factors involved in the therapy of AGU.

2. Materials and methods

2.1. Enzyme preparation and cell lines

Human leukocyte GA was purified 4600-fold (specific activity 96 mU/mg protein) as described by [10]. Normal lymphocytes and fibroblasts were derived from a healthy person and the GA-deficient cells from a Finnish AGU patient carrying the Cys163Ser mutation in the GA gene [11]. Epstein–Barr virus (EBV)-transformed B-cell lines were established as described by [12].

2.2. Treatment of AGU lymphocytes with purified leukocyte GA

EBV-transformed AGU lymphocytes ($5–6\times10^6$) were cultured in 6 ml of RPMI/15% fetal calf serum (FCS) medium [2,12] for 10 days

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Abbreviations: AGU, aspartylglycosaminuria; EBV, Epstein–Barr virus; FCS, fetal calf serum; GA, glycosylasparaginase; GlcNAc-Asn, aspartylglucosamine; PBS, phosphate-buffered saline

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in the presence of various amounts of purified human leukocyte GA. For biochemical analyses, $3–5\times10^6$ cells were withdrawn from the culture, washed twice with phosphate-buffered saline (PBS) and lysed by freezing and thawing in 0.5 ml of 50 mM Na-K-phosphate, pH 7.2, containing 0.1% Triton X-100.

The duration of the therapeutic effect was studied by cultivation of 25×10^6 transformed AGU lymphocytes in the presence of 1000 mU/l of human leukocyte GA in 30 ml of RPMI/15% FCS medium for 9 h, after which 5×10^6 cells were collected and washed three times with 5 ml of PBS. Cultivation was continued in a culture medium in the absence of GA supplementation for 13 days. 5×10^6 cells were withdrawn from the culture on the day stated and they were treated for the biochemical analyses as described above.

2.3. Co-cultivation of normal and AGU cells

A total of 10×10^6 transformed normal (female) and transformed AGU (male) lymphocytes were co-cultured in a cell mixture originally containing 20%, 40%, 60%, 80% or 100% of normal lymphocytes in 20 ml of RPMI/15% FCS medium for 10 days. 5 ml of the cell suspension ($2.5–10\times10^6$ cells) was withdrawn from the each culture for biochemical analyses and another 5 ml for the determination of the number of the Y-chromosome-containing (AGU) cells. 10 ml of fresh medium was added. Cell-to-cell interaction was prevented by culturing 2×10^6 transformed AGU lymphocytes and an equal amount of transformed normal lymphocytes separated by a semi-permeable membrane (Nunc 25 mm tissue culture insert, 0.4 μm polycarbonate membrane) in 8 ml of medium in a 6 well multidish. During the culture, cell samples containing $1–4\times10^6$ cells were collected for the biochemical analyses and treated as described above. An equivalent volume of fresh medium was added. Effect of mannose-6-phosphate in the cell culture medium was studied by culturing 1×10^6 EBV-transformed normal lymphocytes with 4×10^6 transformed AGU lymphocytes in 10 ml of RPMI/15% FCS medium in the presence of 5 mM mannose-6-phosphate (D-mannose-6-phosphate, Sigma Chemical Co., St. Louis, MO, USA) for 10 days. 5 ml of the cell suspension was withdrawn from the culture every second day for the determination of GA activity. 5 ml of fresh M6P medium was added.

Human AGU fibroblasts were cultured [13] to near confluence in a 9.2 cm Petri dish. 2×10^6 transformed AGU or transformed normal lymphocytes were added to the fibroblast culture in 20 ml RPMI/15% FCS and the cultivation was continued for 6 days. Fibroblasts were analyzed by immunofluorescence microscopy.

2.4. Biochemical analyses and Y-chromosome hybridization assay

GA activity was measured with a fluorometric assay using β-(7-amido-4-methylcoumarin)-L-aspartic acid (Bachem Feinchemikalien AG, Bubendorf, Switzerland) as a substrate [14]. One unit of the enzyme causes a loss of 1 μmol of substrate per minute under standard conditions. The GlcNAc-Asn amount in the cell homogenates was determined by high-performance liquid chromatography using carboxymethylcysteine (Sigma Chemical Co., St. Louis, MO, USA) as an internal standard [15]. Protein concentration was measured with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA, USA) according to the manufacturer's instructions.

Y-chromosome-containing AGU lymphocytes were determined using Oncor Rapid In Situ Hybridization kit (Oncor, Inc., Gaithersburg, MD, USA). Hybridization with a fluorescein-labeled Y-chromosome-specific DNA probe was performed according to the manufacturer's instructions.

2.5. Immunofluorescence microscopy

After 6 days of co-cultivation of transformed normal lymphocytes and AGU fibroblasts, the cell culture medium and lymphocytes were removed. The fibroblasts were washed with PBS and incubated for 10 min in the presence of 100% methanol. The cells were washed with PBS and treated with 1% Triton X-100 in PBS for 10 min [16]. The permeabilized cells were blocked with fetal bovine serum and incubated with affinity-purified GA antibodies produced in hens [2]. After washing with PBS, the cells were incubated with hen anti-rabbit antibodies, washed with PBS and incubated with fluorescein isothiocyanate-conjugated anti-rabbit antibodies. After washing, the preparations were mounted with 50% glycerol in PBS containing 1% *N*-propylgallate.

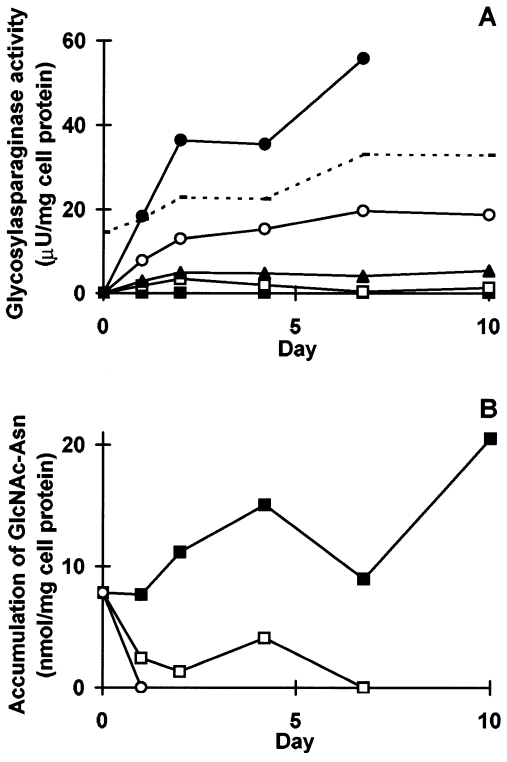

Fig. 1. Uptake of human leukocyte GA by EBV-transformed AGU lymphocytes and accumulation of GlcNAc-Asn in the cells. A: GA activity in the cells during culture in media containing 0 mU/l (■), 10 mU/l (□), 30 mU/l (▲), 100 mU/l (○), 300 mU/l (●) of leukocyte GA ($n=1$). GA activity in normal control lymphocytes cultured without enzyme supplementation (dashed line). B: Intracellular accumulation of GlcNAc-Asn during culture in media containing 0 mU/l (■), 10 mU/l (□) and 100 mU/l (○) of leukocyte GA ($n=1$).

3. Results

3.1. Treatment of AGU lymphocytes with purified leukocyte GA

Human leukocyte GA activity was relatively stable in the cell culture medium at 37°C with the half-life of 2.5 days (data not shown). By incubation of EBV-transformed AGU lymphocytes in the presence of 300 mU/l of purified human leukocyte GA in culture medium, the GA activity present in transformed normal lymphocytes (17.7 μU/mg cell protein) was attained in the diseased cells within 24 h (Fig. 1A) and the concentration of GlcNAc-Asn in the cells was normalized (Fig. 1B). When the culture medium contained 10–100 mU/l of the leukocyte enzyme (Fig. 1A), the normal GA activity was not achieved in the AGU cells, but the accumulation of GlcNAc-Asn in them was prevented during a prolonged cultivation (Fig. 1B). 10 mU/l of leukocyte GA in the medium brought the GA activity in the AGU lymphocytes up to the mean value of 0.8 μU/mg cell protein (0.3–1.3 μU/mg; $n=2$) (Fig. 1A) corresponding to 2–7% of the mean activity in normal lymphocytes (19.4 μU/mg cell protein, range 8.1–32.0 μU/mg; $n=8$) and the metabolism of GlcNAc-Asn in the cells was completely corrected after 7 days of culture (Fig. 1B).

Cultivation of transformed AGU lymphocytes for 9 h in the presence of 1000 mU/l of GA restored the GA activity in the target cells to nearly the level present in normal lymphocytes (Fig. 2A). When the cultivation was continued in the culture

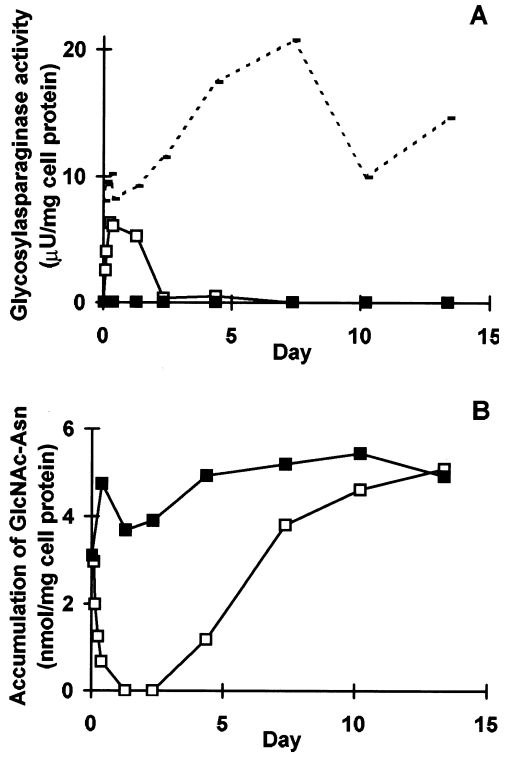

Fig. 2. GA activity and GlcNAc-Asn metabolism in EBV-transformed AGU lymphocytes following treatment with leukocyte GA. The transformed AGU cells were cultured for 9 h in the presence of 1000 mU/l of leukocyte GA. After washing, the cultivation of the cells was continued in culture medium without GA supplementation. A: GA activity. B: Accumulation of GlcNAc-Asn in the cells during culture. GA-treated AGU lymphocytes (□), AGU lymphocytes without enzyme treatment (■) and normal control lymphocytes without enzyme treatment (dashed line) ($n = 1$).

medium without GA supplementation, the intracellular GA activity dropped from its highest value to the level of untreated cells within 7 days. The half-life of GA in transformed AGU lymphocytes was estimated to be approximately 1 day. The intracellular GlcNAc-Asn concentration was normalized within the first 24 h of the experiment (Fig. 2B) and re-accumulation of the compound was detected on day 4, when the GA activity in the cells had decreased to 0.5 μU/mg cell protein. The concentration of GlcNAc-Asn reached the untreated level in the cell mixture within 13 days of culture.

3.2. Treatment of AGU lymphocytes and fibroblasts by co-cultivation with normal lymphocytes

In a cell culture mixture, which contained 80% of transformed normal and 20% of transformed AGU cells, the metabolism of GlcNAc-Asn was completely corrected within 24 h (Fig. 3). In the cell mixture, which originally contained 40% of transformed AGU lymphocytes and 60% of transformed normal cells, the GlcNAc-Asn metabolism was completely normalized on the second day in culture (Fig. 3), in the presence of 65% of normal cells. In the cell pool, which contained 60% of transformed AGU lymphocytes in the beginning of the experiment, the GlcNAc-Asn concentration decreased below the detection limit (less than 0.3 nmol/mg cell protein) on day 8 (Fig. 3). Based on Y-chromosome assay, the cell pool contained 65% of transformed normal cells at that time. In the cell mixture, which initially contained 80% of transformed

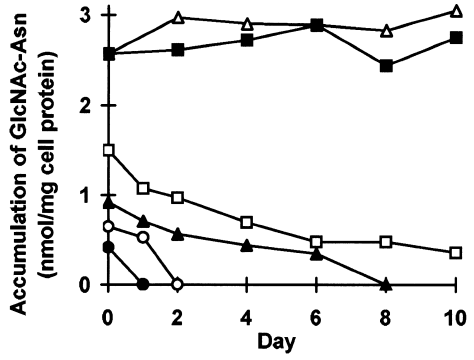

Fig. 3. Correction of the metabolic defect in EBV-transformed AGU lymphocytes and effect of cell-to-cell interaction in the transfer of GA to transformed AGU lymphocytes during co-cultivation with EBV-transformed normal lymphocytes. Accumulation of GlcNAc-Asn in the cell mixture during co-cultivation of normal and AGU lymphocytes for the indicated times in medium containing 80% (□), 60% (▲), 40% (○) or 20% (●) of AGU cells in the beginning of the experiment ($n = 1$). The mean GlcNAc-Asn concentration in transformed AGU lymphocytes which were separated from an equal amount of transformed normal lymphocytes by a semi-permeable membrane during culture (△) compared to GlcNAc-Asn content in control AGU cells (■) ($n = 2$).

AGU cells, a small amount of GlcNAc-Asn was still left in the diseased cells on the tenth day of culture (Fig. 3), although the proportion of transformed normal lymphocytes was increased to 60%. The correction of the metabolic defect during co-cultivation was not inhibited by 5 mM mannose-6-phosphate (Fig. 4). In a cell mixture, which contained 20% of transformed normal and 80% of transformed AGU lymphocytes, the GA activity reached, on day 6, the level present in normal lymphocytes in the presence or absence of 5 mM mannose-6-phosphate (Fig. 4), indicating that mannose-6-phosphate does not inhibit the correction of the GA activity.

When equal amounts of transformed AGU and transformed normal lymphocytes were separated by a semi-permeable membrane and cultured for 10 days, no decrease in the GlcNAc-Asn content (Fig. 3) was observed in the AGU cells. The GA activity in the cell culture medium remained between 1.03 and 1.14 μU/ml indicating that lymphocytes do not excrete significant amounts of the enzyme.

The transfer of GA from transformed normal lymphocytes

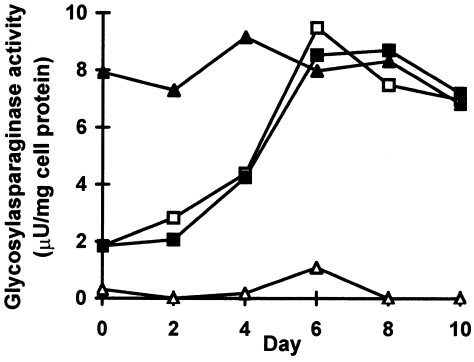

Fig. 4. Effect of mannose-6-phosphate on cell-to-cell transfer of GA. EBV-transformed AGU lymphocytes were cultured with transformed normal lymphocytes in the presence (■) or absence (□) of 5 mM mannose-6-phosphate for 10 days. Transformed normal lymphocytes (▲) and AGU lymphocytes (△) without mannose-6-phosphate ($n = 1$).

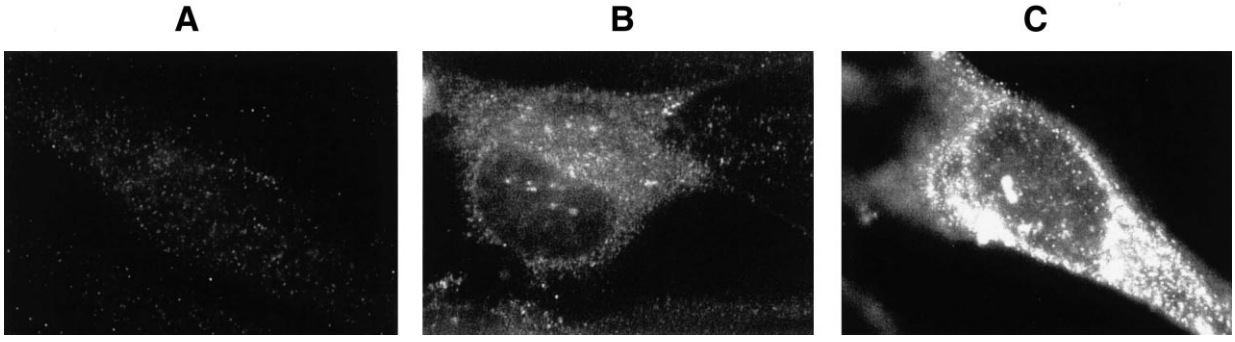

Fig. 5. Immunofluorescence micrographs of GA in AGU and normal fibroblasts. A: GA-deficient fibroblasts. B: Fibroblasts from a normal control person. C: GA-deficient fibroblasts after co-culture with EBV-transformed normal lymphocytes for 6 days.

to AGU fibroblasts during co-cultivation was studied by immunohistochemistry. After 6 days of co-cultivation, the presence of immunoreactive GA could be observed in the AGU cells by immunofluorescence microscopy (Fig. 5C). The immunoreactive GA showed a similar distribution pattern in the treated cells as in normal human fibroblasts (Fig. 5B,C), indicating that GA had been predominantly targeted to the lysosomes of the cells.

4. Discussion

The mannose-6-phosphate receptor-mediated endocytosis [2] of exogenously administered human leukocyte GA effectively corrects the defect in glycoprotein metabolism in transformed AGU lymphocytes. When the enzyme activity in AGU cells is above 7% of that present in transformed normal lymphocytes, accumulation of GlcNAc-Asn is prevented in the diseased cells, which is comparable to the findings with recombinant GA [2,17]. Treatment for 9 h with 1000 mU/l of GA corrected the GlcNAc-Asn metabolism in the transformed AGU cells within 24 h whereas re-accumulation of the compound to the untreated level in the cells took almost 2 weeks. The high therapeutic efficacy of leukocyte GA and slow re-accumulation of GlcNAc-Asn in the treated cells are encouraging findings for developing enzyme replacement therapy for AGU.

AGU lymphocytes or fibroblasts can also be treated by co-cultivation with normal lymphocytes. The transfer of GA requires cell-to-cell interaction to permit the exocytosis of the correcting enzyme from normal lymphocytes with appropriate endocytosis into abnormal ones. The mechanism of the direct cell-to-cell transfer of GA does not involve mannose-6-phosphate receptor-mediated endocytosis, since it was not inhibited by 5 mM mannose-6-phosphate. The comparable lack of inhibition of mannose-6-phosphate has also been demonstrated in cell-to-cell transfer of an other lysosomal enzyme, β-glucuronidase [18]. The immunofluorescence staining pattern of GA in fibroblasts acquired by cell-to-cell transfer was similar to that of normal GA transfected in COS-1 or CHO fibroblasts [19,20], indicating that the enzyme is predominantly targeted to the lysosomes of AGU fibroblasts and that mannose-6-phosphate receptor-mediated transport might be involved inside the cells to take the enzyme into lysosomes. The cell-to-cell transferred GA is catalytically active and degrades the storage compound in the recipient AGU lymphocytes. The efficacy of the cell-to-cell transfer of GA in the correction of the metabolic defect in those cells is related to the proportion of normal lymphocytes in the cell mixture, since normal lymphocytes do not excrete GA outside of cells.

The precise mechanism of the contact-mediated enzyme transfer is not clear. It is known that cell contact involves specific adhesion molecules, which can evoke different cellular activation pathways. It is suggested that cell-to-cell contact induces synthesis of new lysosomal enzyme precursors, which are subsequently transferred to the recipient cells in non-coated vesicles through the endocytic pathway [21]. The direct transfer of enzymes via cell-to-cell contact is restricted to only certain lysosomal enzymes [22]. Although the transfer of lysosomal enzymes via direct cell contact is the mechanism mostly utilized by cell types which do not secrete enzymes, it is also used by cells secreting the enzymes in addition to the receptor-mediated uptake [18]. The contact-mediated transfer of lysosomal enzymes from lymphocytes has an important role in enzyme replacement therapy by bone marrow transplantation of lysosomal diseases [6,23]. This applies also to AGU, since a technically successful bone marrow transplantation has beneficial effects on the natural cause of the disease [9].

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