Feasibility of using cryopreserved lymphoblastoid cells to diagnose some lysosomal storage diseases

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Abstract

Objectives: The Epstein–Barr virus (EBV) is utilized as a tool in the study of cellular biology because of its capacity to transform B-lymphocytes. For this reason, EBV is used in conservation of human B-lymphocytes for long periods for subsequent evaluation of lysosomal hydrolase activity. Lymphoblastoid cell lines have several advantages for use over other cell types, such as prompt availability and possibility to develop, characterize and standardize cell banks, to test effects of promising pharmaceutical reagents. The study below presents biochemical data that demonstrate validity of lymphoblastoid cell lines for diagnosis of GM1-gangliosidosis, Gaucher, Fabry and Pompe diseases and mucopolysaccharidosis type I.

Materials and methods: Cultures were prepared from peripheral blood, collected from 25 normal subjects and 13 affected individuals. Enzyme activities and immunohistochemistry (IHC) were measured. Activities of enzymes β -galactosidase, β -glucosidase, α -iduronidase, α -galactosidase and α -glucosidase were measured before and after cryopreservation for 180 days. Enzymatic activity was measured when transformation was confirmed by IHC.

Results: We observed some significant alterations in enzymatic activity of non-cultured cells when compared to others that had been cultured for 12 days and kept frozen for 180 days.

Conclusions: However, these alterations did not invalidate use of the technology of transformation of lymphoblastoid cell lines with EBV, to diagnose the

diseases mentioned above, in view of the fact that the cultured cells, before and after freezing, demonstrated similar enzymatic activities.

Introduction

The Epstein–Barr virus (EBV) is often used as a tool in cell biology studies due to its capacity to transform B-lymphocytes (1). B-lymphocytes undergo a series of morphological changes during the transformation process including the formation of cytoplasmatic vacuoles rich in lysosomes (2). For this reason, EBV is used also as a tool in studies of evaluation of activity of human lysosomal acid hydrolases (3).

Lysosomal storage diseases (LSD) form a group of disorders caused mainly by deficiency of an acid hydrolase. LSD diagnosis is based on determination of enzymatic activity. In this article, we study five diseases, namely GM1 gangliosidosis (β -galactosidase deficiency), Gaucher disease (β -glucosidase deficiency), mucopolysaccharidosis type I (α -iduronidase deficiency), Fabry disease (α -galactosidase deficiency) and Pompe disease (α -glucosidase deficiency). These diseases are prevalent in Brazil (4) and currently all are treatable, except GM1 gangliosidosis (5,6).

Although determination of deficient lysosomal acid hydrolases can also be performed in skin fibroblasts, lymphoblastoid cell lines (LCLs) have several advantages over skin fibroblast lines: (i) they are more readily obtained, (ii) suffer considerably lower cytogenetic variation, (iii) undergo faster cell proliferation combined with a long lifespan, (iv) produce much more cellular material and (v) are more easily re-established in culture after being frozen (1,3). These advantages enable fast and reliable diagnosis of the diseases described (7,8) and facilitate creation of well-characterized and standardized cell banks to test effects of promising pharmaceutical agents such as chaperones and substrate inhibitors.

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In a previous study (9) we have demonstrated that LCLs could be established with 100% efficiency and cryopreservation, on day 12 after infection with EBV, by performing a 3-(4,5-dimethyl 2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, to examine cell proliferation during EBV transformation. Moreover, we found that activity levels of enzymes β -galactosidase, β -glucosidase and α -iduronidase measured in LCLs before and after cryopreservation in liquid nitrogen for 30 days, remained constant.

This study investigated effects of both EBV transformation and long-term frozen storage (180 days in liquid nitrogen) on hydrolytic activities of five lysosomal enzymes from lymphocytes and LCLs.

Materials and methods

Collection of peripheral blood and isolation of mononuclear cells

Twenty millilitres of peripheral blood was collected from 25 healthy adults (34.88 ± 10.45 years; 19–55 years) and from 13 patients with Gaucher disease, mucopolysaccharidosis type I and Fabry disease (31.66 ± 10.34 years; 19–45 years) by venipuncture. Blood samples were stored in lithium–heparin tubes for analysis. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over a Ficoll-Hypaque (Sigma, St Louis, MO, USA) gradient (900 g, 30 min). Cells were counted microscopically ($\times 100$), and viability was always found to exceed 95%, as judged from their ability to exclude trypan blue (Sigma). A control sample of approximately 9×10^6 cells was stored at -40°C for ultrastructural analysis.

The study protocol was approved by both scientific and ethics committees of the HCPA (*Hospital de Clínicas de Porto Alegre*, Porto Alegre, Brazil), and written informed consent was signed by all subjects.

EBV stock preparation

Virus stock was obtained from an EBV-transformed B95-8 marmoset cell line (Rio de Janeiro Cell Bank, Federal University of Rio de Janeiro, Brazil). Cells were grown in complete RPMI-1640 medium, and cultures were maintained for 18–20 days. On day 7, one fraction of cells was washed (400 g, 10 min) and frozen in liquid nitrogen for storage and cellular lineage maintenance, remaining material was retained in culture for 18–20 days. At the end of this period, the material was filtered through a $0.22\ \mu\text{m}$ membrane (Millipore, Bedford, MA, USA) and stored at -40°C until use.

Transformation of lymphocytes

PBMCs were cultured to a final concentration of 9×10^6 cells/ml in plastic flasks ($25\ \text{cm}^2$, Corning, New York, NY, USA) containing 3 ml of complete medium (RPMI-1640 supplemented with gentamicin 0.5%, fungizone 0.1%, glutamine 1%, Hepes 1% and foetal calf serum 10%; all from Sigma). One millilitre of supernatant derived from the EBV stock preparation and 3 μl of cyclosporin A (2 $\mu\text{g}/\text{ml}$; Sandimmun, Sandoz, East Hanover, NJ, USA) were added to the PBMCs. Transformation by EBV occurs with greater efficiency if the immune T cells are either physically removed from culture or are functionally inactivated (with cyclosporin A, for example) (10). Cells were cultured for 12 days at 37°C in 5% CO_2 in accordance with previous studies (11). LCLs were sub-cultured every 4 days.

Immunohistochemistry assay

To detect EBV-specific proteins in paraffin wax-embedded cells, monoclonal antibody against latent membrane protein 1 (LMP-1) (Dako Denmark A/S, Glostrup, Denmark) was used. LMP-1 is a constitutively activated growth factor receptor encoded by the EBV genome that is responsible for cellular transformation. Its presence in the plasma membrane of EBV-infected cells makes it a useful marker for identifying transformed cells. Antibody location was visualized using streptavidin–biotin–peroxidase complex (Dako Denmark A/S, Glostrup, Denmark) and the diaminobenzidine substrate (DAB)/ H_2O_2 staining method (12).

Assessment of lysosomal acid hydrolases

Analyses of human lysosomal acid hydrolases were performed in lymphocytes and LCLs on the 12th day of culture and after 180 days of freezing. β -glucosidase activity was evaluated using a well established method (13) that utilises 4-methylumbelliferyl- β -D-glucoside (Sigma) as the synthetic substrate, together with sodium taurocholate (Sigma), under standard conditions (pH 5.5 and 37°C). Activities of enzymes β -galactosidase (1) and α -iduronidase (14) were estimated by incubation with 4-methylumbelliferyl- β -D-galactoside (Sigma) and 4-methylumbelliferyl-iduronide (Sigma) as substrates, respectively, under standard conditions (pH 4.0 and pH 2.8 respectively, at 37°C). Furthermore, α -galactosidase activity was evaluated according to a further classical method (15) that uses 4-methylumbelliferyl- α -D-galactoside (Sigma) as synthetic substrate together with citrate phosphate (Sigma), under standard conditions (pH 4.8 and

37 °C). Activity of the enzyme α -glucosidase was evaluated according to one more method (16), which uses 4-methylumbelliferyl- α -D-glucoside (Sigma) as the synthetic substrate, together with acetate (Sigma) in standard conditions (pH 4.0 and 37 °C). Fluorescence was evaluated by spectrofluorometry (450 and 365 nm). Data are expressed as nmol/h/mg of protein (17).

Statistical analysis

Data are reported as mean $\pm$ SD. One-way ANOVA followed by the Tukey test was used to compare enzymatic results of analysis of lymphocytes with those of both fresh and frozen LCLs. Three combinations were performed: lymphocytes versus fresh LCLs, lymphocytes versus frozen LCLs and fresh LCLs versus frozen LCLs. Analysis was performed using statistical software package SPSS 11.5 (SPSS Inc., Chicago, IL, USA), and level of significance was set at $P < 0.05$.

Results

Evaluation of cellular transformation

IHC results are shown in Fig. 1. Positive signals were located over cytoplasm of cells transformed by EBV. It can be observed that in non-transformed cells, there was no reaction between LMP1 antibody and avidin (Fig. 1a). After 12 days of culture with EBV, presence of cell pleio-

morphism and numerous cytoplasmic vacuoles were observed, indicating a positive reaction (Fig. 1b, arrows), mainly along plasma membranes (18).

Furthermore, cell clusters were formed during the 12-day culture. As described previously, these clusters demonstrate successful transformation (Fig. 2).

Evaluation of lysosomal acid hydrolases

Activities of enzymes β -galactosidase, β -glucosidase, α -glucosidase, α -iduronidase and α -galactosidase of normal subjects were measured after isolation of lymphocytes, on day 12 and after 180 days cryopreservation and after isolation, and on day 12, in cells of affected individuals. No change was detected in activities of β -galactosidase and α -glucosidase (Table 1) of normal subjects ($P = 0.309$ and $P = 0.521$ respectively).

Activity of β -glucosidase in isolated lymphocytes was 1.4 times lower than activity measured in LCLs after the 12-day culture period with EBV and to that of LCLs after 6 months cryopreservation, with the difference being statistically significant ($P < 0.0095$) (Table 1).

In patients with Gaucher disease (Table 1), it was observed that isolated lymphocytes did not differ in β -glucosidase activity when compared with LCLs after the 12-day culture period ($P = 0.16$). However, when we compared β -glucosidase activity of isolated lymphocytes of these patients with controls, a statistically significant increase of 16.5 times ($P < 0.001$) was observed in the

Figure 1. Immunohistochemistry: (a) mononuclear cells not yet transformed (day 0); (b) Cells transformed by EBV (day 12); arrows indicate reaction of LMP-1 antibody with DAB, visualized with the characteristic brown colour. Micrographs obtained using an inverted microscope ($\times 1000$).

Figure 2. Visualization of transformed B lymphocytes infected with EBV. PBMCs were incubated with EBV for 12 days. 4- (a) and 12-day (b) cultures were photographed using an inverted microscope (100 $\times$). Increase in cell cluster size indicates success of B-cell transformation.

Table 1. Lysosomal acid hydrolase activities (nmol/h/mg of protein) in lymphocytes and LCLs before and after cryopreservation

Enzymes	Lymphocyte without EBV	LCL before freezing (12-day culture)	LCL after freezing (180 days)
β -galactosidase			
Controls ($n = 25$)	183 $\pm$ 49.7	156.6 $\pm$ 56.4	179.7 $\pm$ 64.7
β -glucosidase			
Controls ($n = 25$)	21.4 $\pm$ 7.2	30.0 $\pm$ 9.8 ^a	29.4 $\pm$ 9.0 ^a
Patients ($n = 3$)	1.3 $\pm$ 1.0 ^b	2.5 $\pm$ 0.2 ^b	
α -glucosidase			
Controls ($n = 25$)	4.9 $\pm$ 1.7	4.3 $\pm$ 1.9	4.9 $\pm$ 2.3
α -iduronidase			
Controls ($n = 25$)	44.4 $\pm$ 14.1	30.5 $\pm$ 8.3 ^a	33.9 $\pm$ 15.8 ^a
Patients ($n = 6$)	0.22 $\pm$ 0.18 ^b	1.5 $\pm$ 0.4 ^{a,b}	
α -galactosidase			
Controls ($n = 25$)	39.2 $\pm$ 10.4	46.1 $\pm$ 17.7	53.9 $\pm$ 21.5 ^a
Patients ($n = 4$)	3.1 $\pm$ 0.7 ^b	3.2 $\pm$ 2.9 ^b	

Data are expressed as mean $\pm$ SD.

^aStatistically different from lymphocyte without EBV.

^bStatistically different from the control group.

latter group. The same type of activity occurred for LCLs after a 12-day culture period (12-fold increase; $P < 0.001$).

Table 1 indicates α -iduronidase activity of controls and MPS I patients. In the control group, activity in isolated lymphocytes increased significantly ($P < 0.004$), 1.45 times higher than that measured after 12 days in culture with EBV and 1.31 times higher in relation to activity measured after 6 months cryopreservation. In patients, we noted that LCL activity after 12-day culture period was significantly higher (6.8 times, $P < 0.002$) compared to α -iduronidase activity from lymphocytes. Activities in lymphocytes and in 12-day LCL were statistically different (201.8 times, $P < 0.001$; 20.3 times, $P < 0.001$, respectively) between control individuals and patients.

Finally, α -galactosidase activity in normal individuals cells (Table 1), after 6 months cryopreservation increased significantly (1.4 times, $P < 0.041$) when compared to activity of the enzyme in isolated lymphocytes. Yet, this was not observed with cells of Fabry disease patients, for whom lymphocyte α -galactosidase activity was similar to that of LCLs after the 12-day culture period ($P = 0.836$). Cells of Fabry disease patients exhibited lower α -galactosidase activity than control cells (lymphocytes: 12.1 times, $P < 0.001$; LCL after 12 days: 14.4 times, $P < 0.001$).

Discussion

In research and diagnosis of LSD, use of LCLs holds considerable importance because these cells are more readily

obtainable, produce expressive amounts of cellular material and are useful in creation of cell banks (1,3). Samples can be conserved in good condition for measurement of enzyme activity at any future time, that is in case of necessity to repeat any previously conducted assay after patient death, or for confirmation of diagnosis.

In a previous study (9) our group observed that some LCL lysosomal hydrolases maintained their activities after freezing in liquid nitrogen for 30 days. This information is very important in cell bank development strategies, but the period studied (30 days) is not sufficient. Here, we tested other cryopreservation periods and compared enzymatic activity of patients with enzymatic activities in cells from normal people. During the first 12 days of culture, LCLs of 25 controls and of 13 patients were produced, indicating that the EBV-induced transformation was 100% successful. This finding agreed with our previous results (9). Efficiency of transformation of the B cells was checked every 4 days by observing formation and increase in clusters and cell counts. We observed changes in size and 'graininess' of lymphocytes when viewed using an inverted microscope (data not shown).

In this study, we observed formation of cell clusters and successful infection using the immunohistochemical technique (Fig. 1a,b). In the first event, LMP1 was not produced as the lymphocytes had not been transformed. However, Fig. 1b shows triggering of a positive reaction (arrows), which demonstrates efficiency of cellular transformation by EBV.

Once the transformation of cells by EBV has been confirmed, it is possible to measure the lysosomal hydrolases activity and verify whether these enzymes remain unaltered after 6 months of cryopreservation.

Samples of lymphocytes recently collected from normal individuals or affected patients, not submitted to treatment with EBV, showed that the activity of the five enzymes studied agreed with those described in the literature (2,19–22).

When lymphocytes of normal cells were submitted to transformation by EBV, a significant alteration was observed in activities of the β -glucosidase and α -iduronidase after 12 days in culture, while activities of the other enzymes remained unchanged. With regard to α -galactosidase activity, a significant shift occurred after 180 days freezing. Enzymatic activity may have been altered by purity of the sample (for example, presence of non-monocytic cells) and/or composition (mixture of monocytes, B cells and T cells) of lymphocytes at different stages of preparation (freshly prepared, isolated, transformed and cultured, frozen). Differences in enzymatic activity among lymphocytes, leucocytes and LCLs have also been observed also in other investigations (7,23). This demonstrates that EBV can influence activity of some

enzymes, which makes it necessary to establish reference levels of enzymatic activity for cells being cultured and immortalized by the virus.

Although EBV, when introduced into the culture medium, altered activity of these three enzymes, this change was not enough to impair the diagnosis of Gaucher disease, mucopolysaccharidosis type I and Fabry disease. Even an increase in activity – as obtained in this study – would not be capable of masking the diagnosis. If activities of these three enzymes in patients, however, were 5% to 10% of normal, a safe, reliable diagnosis would not be possible.

The other objective of this study was to verify whether activities of these five enzymes would have changed after 180 days freezing. If enzymes in cells transformed with EBV could still be used even after several days freezing in liquid N₂, those cells would be useful in cell bank development strategies to test effects of promising pharmaceutical reagents (for example, chaperones, substrate inhibitors, stop-codon read-through drugs), to evaluate different diagnostic substrates, in a standardized manner, or to investigate side effects of substrate accumulation in a uniform cell type (B lymphoblasts).

Results obtained in the present study show that there was no change in enzymatic activity after 6 months cryopreservation. This finding illustrates the importance of cryopreservation when the aim is to maintain material unadulterated over long periods.

Similar results have been previously obtained by our group (9) for β -galactosidase, β -glucosidase and α -iduronidase. Similarly here, we found no differences in activities of these three enzymes after a 30-day cryopreservation period.

In conclusion, presence of the characteristic brown final reaction product observed in the cells after IHC, after the 12-day culture period, was a consistent indication that the lymphocytes had indeed been transformed by the EBV. This transformation was capable of maintaining activity of the enzymes (β -galactosidase, β -glucosidase, α -galactosidase, α -glucosidase and α -iduronidase) even after 180 days cryopreservation. Our study demonstrates the feasibility of use of LCLs as a tool for biochemical diagnosis of five lysosomal storage diseases (gangliosidosis GM1, Gaucher, Fabry and Pompe diseases and mucopolysaccharidosis type I), which are all found frequently in our country, Brazil (4).

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