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Single-cell analysis avoids sample processing bias

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Abstract

Microscale separation tools such as capillary chromatography and capillary electrophoresis (CE) allow the study of metabolism in individual cells. In this work, we demonstrate that single-cell analysis describes metabolism more accurately than analysis of cellular extracts. We incubated HT29 cells (human colon adenocarcinoma) with a fluorescently labeled metabolic probe. This disaccharide, LacNAc, was labeled with a fluorescent dye, tetramethylrhodamine (TMR). The probe was taken up by the cells and metabolized to a number of products that retained the fluorescent label. We then split the cells into two batches. A cellular extract was prepared from one batch and analyzed by CE with laser-induced fluorescence (LIF) detection. The cells from the second batch were used for single-cell analysis by CE–LIF. Separation and detection conditions were identical for extract and single-cell analyses. We found that the electropherogram obtained by averaging the results from a number of single cells differed significantly from the cell extract electropherogram. Differences were due to sample processing during extract preparation. Disruption of the cells liberated enzymes that were compartmentalized within the cell, which allowed non-metabolic reactions to proceed. The accumulation of these non-metabolic products introduced a bias in the cell extract assay. During single-cell analysis, cells were lysed inside the capillary and the separation voltage was applied immediately to separate the enzymes from their substrates and prevent non-metabolic reactions. This paper is the first to report that CE analysis of single cells provides more accurate metabolic information than the CE analysis of a cellular extract.

Keywords: Single-cell analysis

1. Introduction

Capillary electrophoresis (CE) has become an important tool in studying cellular chemistry. There are two approaches in such studies, bulk analysis of a large number of cells using cellular extracts [1–4] and single-cell analysis [5–8]. Until recently, bulk analysis was the preferred method of study unless heterogeneity of the cell population was of interest.

In this paper, we consider single cell studies of oligosaccharide metabolism. Glycosylation is an important metabolic process. Being the final step in protein biosynthesis, it plays a crucial role in intracellular recognition and adhesion, cell development and differentiation, hormone recognition and immunoresponse [9,10]. Many pathologies, for example teratogenesis [11] and carcinogenesis [12], are associated with abnormal glycosylation. CE has been used for bulk metabolic analysis of glycosylation in cancer cells [13–17]. In our studies, tetra-

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methylrhodamine (TMR) labeled metabolic substrate, a disaccharide or a trisaccharide, was introduced into cancer cells where it was converted to a number of metabolites [15–17]. Cellular extracts were prepared from a large number of cells. This multi-step procedure involved removing cellular debris and hydrophilic cellular components followed by concentrating the hydrophobic TMR-labeled species. The extract was then analyzed by CE–LIF. Such an approach was very useful since it allowed for simultaneous analysis of multiple glycosylation and glycolysis activities.

We have recently developed CE techniques for the study of glycosylation and glycolysis in individual cancer cells [18]. This technique allows us to monitor metabolism at the single-cell level and therefore is called *metabolic cytometry*.

In the present study, we compare electropherograms obtained by metabolic cytometry and by the bulk analysis of a cellular extract. We demonstrate that metabolic cytometry provides more reliable metabolic information than bulk analysis. The bulk analysis introduced a bias associated with sample preparation, whereas single-cell analysis greatly reduced sample handling, and thus minimized the possibility of such a bias.

2. Experimental

2.1. Reagents

Unless otherwise stated, all reagents used in this work were commercial chemicals of analytical-grade used without additional purification. Aqueous solutions were prepared using distilled deionized water and filtered with 0.22-μm pore size disposable filters (Millipore, Bedford, MA, USA). The same buffer (10 m*M* phosphate, 10 m*M* phenylboronic acid, 10 m*M* disodium tetraborate and 10 m*M* sodium dodecyl sulfate (SDS) at pH 9.3) was used as the separation buffer and as the sheath flow fluid. Reagents were from Sigma (St. Louis, MO, USA).

2.2. Cell culture procedures

The HT29 cell line was grown to 80% confluence in Dulbecco's modified eagle's medium, supplemented with 10% fetal calf serum and 40 μg/ml gentamicin (all from Gibco BRL, Gaithersburg, MD, USA) at 37°C in a 5% CO_2 atmosphere. The cells were then incubated for 18 h with 25 μ*M* *N*-acetyllactosamine (LacNAc)–TMR [16]. After incubation the cells were washed eight times with phosphate buffered saline (PBS, Sigma) to remove the residual LacNAc–TMR and its metabolic products from the cell medium. The cells were resuspended in PBS and split into two batches. One batch of cells was used for single-cell analysis and the other one for bulk analysis.

2.3. Analytical procedures

For single-cell experiments, the cell suspension was diluted with PBS to a final density of 10^4 cells/ml. We injected a single cell into the capillary with an 11 kPa×1 s siphoning pulse created by a 108-cm long water column. While in the capillary, the cell was lysed within 30 s by the SDS surfactant, which was in the running buffer. The cellular contents were separated using micellar electrokinetic capillary chromatography and TMR-labeled species were detected using LIF of the TMR label at 580 nm.

The procedure used to prepare the extract of TMR-labeled species for bulk analysis have been reported [18]. Briefly, the cell suspension (total: 2×10^6 cells) was diluted with PBS to 5×10^6 cells/ml. Then the cells were homogenized in a microtissue grinder at 0°C with 50 strokes every 15 min for 1.5 h. The sample was centrifuged at 320 *g* for 4 min to remove cellular debris. The supernatant was loaded onto a C_{18} Sep Pak cartridge (Waters). The cartridge was washed with water and TMR-labeled species were eluted from the cartridge with HPLC-grade methanol (Sigma). The methanol was evaporated and the residue was dissolved in 160 μl of the CE running buffer. The extract was injected into the capillary by an 11 kPa×1 s siphoning pulse. Separation and detection conditions were similar to those for single-cell analysis.

2.4. Electrophoresis system

The separation was performed using a locally constructed CE instrument described in detail else-

where [19]. The electrophoresis was driven by a CZE1000R high-voltage power supply (Spellmann, Plainview, NY, USA) at 18 kV. The power supply was controlled with a Macintosh Quadra 650 computer via an NB-MIO-16XH-18 input/output board (National Instruments, Austin, TX, USA). This board was also used to record the electrical current and fluorescence intensity as functions of time during the electrophoretic separation.

Our fluorescence detector used a 1-mW helium–neon laser (Melles Griot, Nepean, ON, USA) emitting at 543.5 nm. The laser beam was focused with 6.3× objective (Melles Griot). The capillary was inserted in a locally constructed sheath-flow cuvette so that the laser beam was approximately 20 μm below the tip of the capillary. The cuvette was similar to one manufactured by Ortho Diagnostics around 1980 (model 300-0511-000). Our cuvette had a 200-μm square flow chamber with 1 mm thick quartz windows. Sheath-flow through the cuvette was created by a simple siphon system, and the sheath-flow buffer was the same as used for the separation.

The polyimide coating was removed from the last ~2-mm portion of the capillary to reduce background fluorescence from the coating. Analyte fluorescence was collected at 90° with respect to the incident laser beam using a 60×, 0.7 N.A. microscope objective Model 60X-LWD (Universe Kogaku, Oyster Bay, NY, USA) and spectrally filtered with interference filter centered at 580 nm and having 30 nm bandwidth (Omega Optical, Brattleboro, VT, USA). Fluorescence was detected with an R1477 photomultiplier tube (Hamamatsu, Bridgewater, NJ, USA).

Standard solutions of LacNAc–TMR were used to calibrate the optical detection system. The mass detection limit of the instrument was determined to be 100 TMR-labeled molecules (30–33).

3. Results and discussion

A typical electropherogram consisted of six peaks for both cell extract and single-cell analysis (Fig. 1). Peaks were identified using CE–LIF by co-injecting the standards. Peaks 1 and 2 were the products of the substrate glycosylation, a tetrasaccharide Le^y–TMR and a trisaccharide Le^x–TMR, respectively. Peak 3

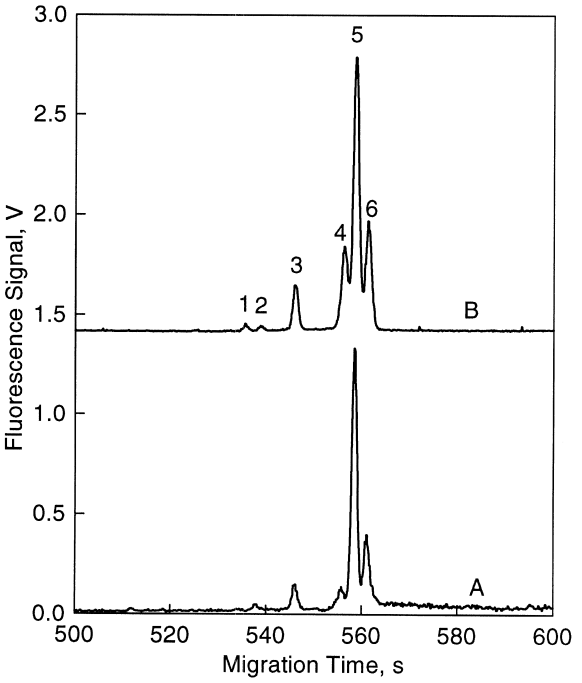

Fig. 1. Electropherograms obtained from (A) single-cell and (B) bulk analysis of the cell extract. The peaks correspond to: (1) a tetrasaccharide, Le^y–TMR; (2) a trisaccharide, Le^x–TMR; (3) unreacted substrate, LacNAc–TMR; (4) unidentified product; (5) a monosaccharide, βGlcNAc–TMR; (6) a TMR–aglycone, $HO(CH_2)_8CONH(CH_2)_2NH$–TMR.

corresponded to the unreacted substrate, LacNAc–TMR. Peak 4 was unidentified. Peaks 5 and 6 were ascribed to the products of substrate hydrolysis, a monosaccharide and TMR–aglycone, βGlcNAc–TMR and ($HO(CH_2)_8CONH(CH_2)_2NH$–TMR), respectively.

Normalized peak heights (N_I) were determined as the ratios between individual peak heights (I_i) and the sum of the heights for all six peaks:

$$N_i = \frac{I_i}{\sum_{k=1}^{6} I_k}$$

Relative standard deviations of the normalized peak heights were higher for single-cell analyses than for cellular extract separations (Fig. 2). This variation reflects the fact that individual cells differ in their ability to accumulate and metabolize Lac-

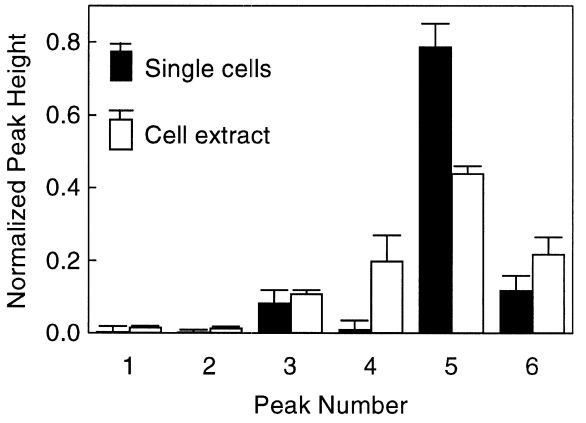

Fig. 2. Average normalized peak heights obtained from single cells ($N=20$) and cellular extract ($N=18$). The peaks correspond to: (1) a tetrasaccharide, Le^{y}–TMR; (2) a trisaccharide, Le^{x}–TMR; (3) unreacted substrate, LacNAc–TMR; (4) an unidentified product; (5) a monosaccharide, βGlcNAc–TMR; (6) a TMR–aglycone, $HO(CH_2)_8CONH(CH_2)_2NH$–TMR.

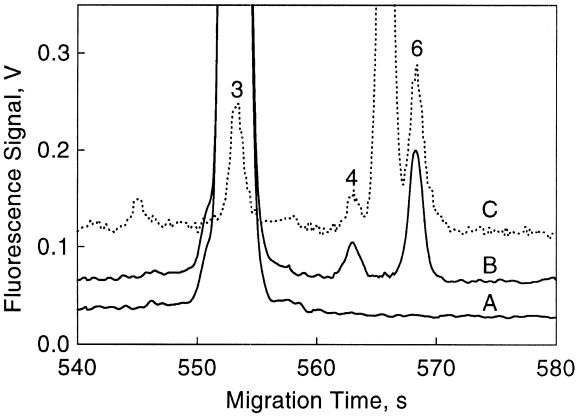

Fig. 3. Glycolysis during extract preparation: (A) LacNAc–TMR and (B) LacNAc–TMR after treatment with cellular homogenate. The formation of products 4 (unidentified) and 6 (TMR–aglycone) indicates that the saccharides are modified during extract preparation. Curve (C) shows the electropherogram obtained by metabolic cytometry as a positive control.

NAc–TMR. We believe that the main reason for the cell-to-cell metabolic variation is the cells' asynchronous growth in cell culture. This assumption is based on the observation that the level of glycosylation depends on the cell cycle phase [18,20].

Comparing the results for single-cell analyses and those for extract analyses revealed one more difference: the average normalized heights for peaks 4, 5 and 6 in single-cell analyses differed significantly from those in the analysis of cellular extracts (see Fig. 2). The relative concentration of product 5 was significantly lower in the extract separations than in the single-cell analyses ($p=9\times10^{-10}$). The relative concentrations of products 4 and 6 were, in contrast, lower in single-cell analyses ($p=2\times10^{-17}$ and $p=2\times10^{-4}$, respecctively). These differences could be due to enzymatic reactions modifying product 5 to products 4 and/or 6 during the extract preparation but occurring minimally in the single-cell analysis.

To confirm this suggestion, we performed a control experiment. Cells were prepared as usual; the only difference was that LacNAc–TMR was not added for the 18 h incubation but added in the very beginning of the extraction procedure instead. By adding LacNAc at the start of the extraction procedure, we ensured that all the enzymatic changes of the substrate were caused by reactions occurring during the extraction procedure. We found that products 4 and 6 were present in the extract in addition to LacNAc–TMR (Fig. 3). This result confirmed that enzymatic reactions proceeded at a finite rate during extract preparation even when carried out at 0°C. Significant differences between average normalized peak heights obtained from extract analyses and those obtained from the single-cell analyses (see Fig. 2) were caused by enzymatic reactions during extract preparation.

To explain the differences between the results of single-cell and extract analyses we hypothesize that product 5 and the enzymes responsible for its modification to products 4 and/or 6 are localized in different cellular compartments. Therefore, extensive transformation of 5 to 4 and 6 is impossible in intact cells. When single cells were analyzed, SDS lysed the cells inside the capillary and separation started a few seconds after lysis. SDS quickly denaturated the enzymes and prevented them from mixing and reacting with their substrates. Therefore, enzymatic reactions that convert product 5 to products 4 and/or 6 did not proceed in the capillary. In contrast, during the extract preparation, all the cellular compartments were mechanically disrupted and their contents were homogenized for 1.5 h. Therefore, the enzymes reacted with product 5 and converted it to products 4 and 6. Not accounting for these reactions introduces

a metabolic bias in interpreting the results. To minimize these reactions, the extract preparation was carried out at 0°C. But even at this low temperature, enzymatic reactions proceeded at a low but finite rate. Adding a surfactant to lyse the cells could also minimize the extent of such reactions. However, surfactants were excluded from the standard extraction procedure because they preconcentrated together with TMR-labeled species on a C_{18} Sep Pak cartridge; excess surfactants interfered with both the CE separation and mass spectrometry detection.

4. Conclusions

One advantage of single-cell analysis is that enzymatic reactions are quenched upon cell lysis; contact with the surfactant denatures the enzymes and application of the electrophoresis potential separates substrates from any surviving enzymes. Thus, metabolic analysis of single cells can provide more accurate information than analysis of cellular extracts. In addition, single-cell analysis, in contrast to extract analysis, allows the study of the heterogeneity of a cell population. It is especially important for cancer cells that are known to be highly heterogeneous in both phenotype and genotype [21,22]. Lastly, single cell analysis might be capable of detecting unstable intermediates with lifetimes corresponding to lysis time.

There are, however, several limitations of single-cell analysis by CE. Single-cell analysis is ideally suited for cell suspensions, such as cultured cells and blood samples. In contrast, the isolation of single cells from tissue samples may involve tedious and time-consuming procedures [23]. Moreover, single-cell analysis relies on rapid cell lysis inside the capillary. Some cells, such as from plants, fungi and yeast, have very robust walls that resist lysis by surfactants; the wall must be removed prior to cell injection into the capillary [24].

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