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Measurement of cell numbers by means of the endogenous enzyme hexosaminidase: applications to cell adhesion assays in endometrial and ovarian carcinoma cell lines

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Summary

Using a chromogenic substrate for the lysosomal enzyme, hexosaminidase to estimate cell nubers, a sensitive, simple, and non-radioactive procedure has been previously developed (1) in which microtiter reaction wells are directly scanned using a spectrophotometer. The applications of this method to quantitation of cell numbers and adhesion assays to coated substrates in endometrial adenocarcinoma (Ishikawa) and epithelial ovarian cancer (Ovcar) cells are discussed. The methods are safe, cheap, and easily applicable to biological researches particularly in developing countries.

Résumé

En faisant usage d'un substrat chromogenique de l'enzyme lysosomale, l'hexosaminidase, afin d'estimer le nombre de cellule, une procedure simple et non-radioactive avait été précédement developé. Dans cette procédure, les plats microtitrés dans les puits desquels se deroulent les reactions sont crible à l'aide d'un spectrophotomètre. Les applications de cette methode pour determiner quantitativement le nombre de cellules et les assays d'adhésions afin de fixer les substrats dans l'adrenocarcinome endometrique (Iskikawa) et l'epithelium ovarien des cellules cancereuses est ici discuté. Ces methodes sont saines, moins chères et facilement applicable aux recherches biologiques particulièrement dan les pays en voie de développement.

Introduction

The enzyme N-acetyl-B-D-hexodaminidase (2-acetamido-2 deoxy -B-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) is a lysosomal enzyme involved in the degradation of glycosylated cellular constituents. It has been shown to exist in many tissues and in different phyla [1].

A p-nitrophenoladerivatized substrate can be employed for the spectrophotometric quantitation of the activity of this enzyme. Its ubiquitous occurrence and catalytic activity make this enzyme an ideal indicator for analysis of cell numbers. Its use in this regard was first described by Koponen *et al.* [2] to score the sensitivity of a series of thymoma cell lines to the growth inhibitory action of cyclophosphorin A. Measurement of this enzyme in heterogenous cell populations with regard to their stage of differentiation and lineage needs to be analyzed with the caveat that different cells may contribute variably to the overall enzyme reaction. With this limitation in mind, a number of assays where an effect upon cell number is sought may be accommodated to measurement of this enzyme.

We describe here its use for the measurement of integrin cell adhesion assays in human endometrial adenocarcinoma cell lines (Ishikawa cell lines) and in epithelial ovarian cancer cell lines (Ovcar 420 and 432).

The methods described allow minimal manipulation of the samples with good exactitude in determination. Also, the methods are non-radioactive; they are not dependent upon expensive or unstable reagents. The advantages make them easily applicable in third world countries.

Materials and methods

Reagents:

The substrate for hexosaminidase, p-nitrophenol-N-acetyl-B-D-glucosaminide (PNAG) was obtained from Sigma (St. Louis, MO). Ovarian cancer cell lines (Ovcar 420 and 432) were generously supplied by Dr. George Oltz (Hershey Medical Center, PA). Biocoat wells for fibronectin, collagen, and laminin were purchased from Collaborative Biomedical Products (Becton Dickinson Labware MA).

Cell culture

All cultures were performed in MEM (minimum essential medium) with 5% fetal calf serum and supplemented with 2 mM L-glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin. The cell lines used were Ishikawa (human endometrial adenocarcinoma) and Ovcar (epithelial ovarian carcinoma) cell lines.

The hexosaminidase reaction

The substrate for the enzyme hexodaminidase, PNAG was dissolved at 7.5 mM in 0.1M citrate buffer, pH5. The solution was then mixed with an equal volume of 0.5% Triton X-100 in water aliquoted and stored at - 20°C. The substrate solution was then added in volumes of 50ul to cells in flat bottom microtiter wells. The plates were then incubated at 37°C in 100% humidity. After an interval of 30 minutes, the colour reaction was developed and enzyme activity blocked by addition of 50 mM glycine buffer, pH 10.4 containing 5 mM EDTA, and 90 μl/well. Absorbance was measured in a Titertek Multiskan at 405 or 450 nm.

Preparation of wells for experiments:

Ninety-six microtiter well plates were used for the experiments. The microtiter plates are either plain plastics used for standard curve assays or those coated with ligands—laminin (LM), fibronectin (FN), collagen (Coll) or vitronectin (VN). Every well was then coated with 1% BSA (250 μl/well) for 1 hour at room temperature. The BSA (bovine serum albumin) was to block spaces in between the ligands. The BSA was then removed and the wells are washed twice with 250 μl of sterile PBS (phosphate buffer solution) per well. A final wash was then done once with Tyrodes Hepes buffer. The buffer was then removed from the wells which were now suitable for the experiments. The cells were then plated in the required concentrations in the 96 microtiter well plates.

Preparation of cells

At near confluency, the Ishikawa cells or Ovcar cells were harvested in 1X Trypsin plus EDTA and centrifuged. The harvested cells were then suspended twice in Tyrodes Hepes buffer with Ca++, Mg++ salts. Centrifugation was carried out on each occasion. Finally the cells were suspended in Tyrodes Hepes buffer with Ca++, Mg++ and BSA. Cells were subsequently plated in the required concentrations in 25 μl of this solution in the microtiter plates.

Plating of cells and experimentation

Plating of cells was done as follows: the cells were plated on the ligands (LM,FN,VN) in the required concentrations in 50 μl Tyrodes/BSA solution while those plated on plastics were in 25 μl Tyrodes/BSA solution. The plated cells were left at room temperature for 1-2 hours. Thereafter, the solutions in the ligands were removed and washed three times with binding buffer at room temperature. The solutions in the plastics were

not sucked off, otherwise the cells would be lost since adhesion of cells is very low on the plastics. To normalize volumes, 25 µl of Tyrodes Hepes/BSA buffer and 25 µl of substrate solution (PNAG) was added to the cells plated on ligands (total 50 µl) while only 25 µl of substrate was added to cells plated on plastics (now making a total of 50 µl). The cells were then left to react with the substrate for 1 hour at room temperature. The reaction with the substrate (PNAG) was stopped with the addition of 75 µl/well of 50 mM glycine pH 10.4 and 5 mM EDTA. Absorbance was then read at 405 or 450 nm as appropriate.

Results

The absorbency of the product of the hexosaminidase reaction is directly proportional to the cell numbers

For analysis of hexodaminidase content, cells were added to microtiter plates (plastics) in serial dilutions to obtain varying concentrations. The cells in the plastic microtiters served as standard measurements. Substrate was subsequently added as outlined previously and the experiment was continued for 1 hour at room temperature. Each experiment was in quadruplicate.

Using the Ishikawa cells, the absorbancy was seen to be directly proportional to the number of cells (concentration) in the well (Fig. 1). In the calculations, absorbancies between 0.1 and 1.2 were used. This allows determination of cell numbers over a 12-fold range for a given time of exposure to the substrate.

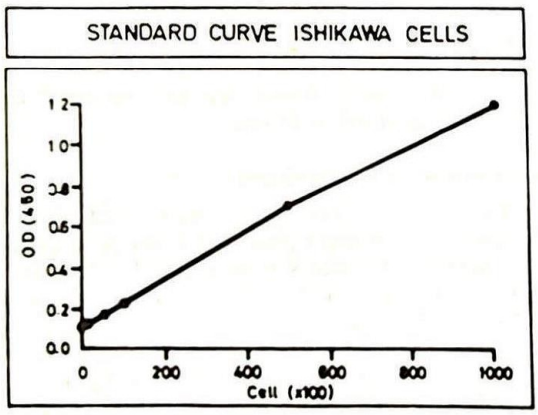

Fig. 1: Relationship between cell numbers and optical density (OD, absorbance) in Ishikawa cells

The epithelial ovarian carcinoma cells (Ovcar 420) also exhibited a direct proportionality between absorbancy and cell number even when fewer number of cells were used as in this case (Fig. 2).

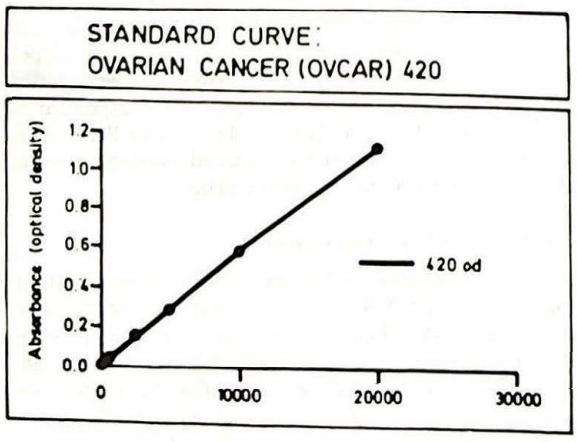

Fig. 2: Relationship between cell numbers and optical density (absorbance) in ovarian cancer cell live (Ovcar 420)

Substrate adhesion of endometrial and epithelial ovarian cancer cells

Adhesion of these cells was measured by incubating the cells for 1 hour at 37°C in microtiter wells that had been coated with ligands (BSA, FN, LM, COLL or VN) for 1 hour at 37°C [1]. The experiments thereafter followed the steps earlier outlined.

Applications

In Ishikawa endometrial adenocarcinoma cells, adhesion assay was done using the obtained standard curve. The experimental conditions remained the same except that 5 x 10^4 cells/well were plated on ligands (BSA, FN, and VN). The results are as shown in Table 1. Percentage cell adherent was then determined by extrapolating from the standard curve; for example, 50,000 cells on BSA gave an OD of 0.083 while 0.713 was the reading on standard. The percentage cell adherent is, therefore, calculated as (.083/.713 x 50,000 = 5820; 5820/50,000 x 100 = 11.6%).

Table 1: Relationship of cell concentration and absorbance in standard and substrate assays

Cell concentration (No of cells/well)	No of cells/walls	Absorbancy (average)
Standard	0	114
	100	115
	500	117
	1000	121
	5000	171
	10,000	228
	50,000	713
	100,000	1 191
Substrate		
+ Fibronectin (FN)		0
+ Vitronectin (VN)		388
+ Bovine serum albumin (BSA		449
		083

+ 50,000 cells/well plated in quadruplicate for each ligand

A similar calculation gave fibronection as 54% adhesion and vitronectin as 63% adhesion (Fig. 3). In ovarian epithelial adenocarcinoma cells (Ovcar 420 and 432), standard curves were similarly determined for each cell line as done in Ovcar 420. The percentage cells adherent to ligands (BSA, LM, COLL, FN) are shown in Fig. 4.

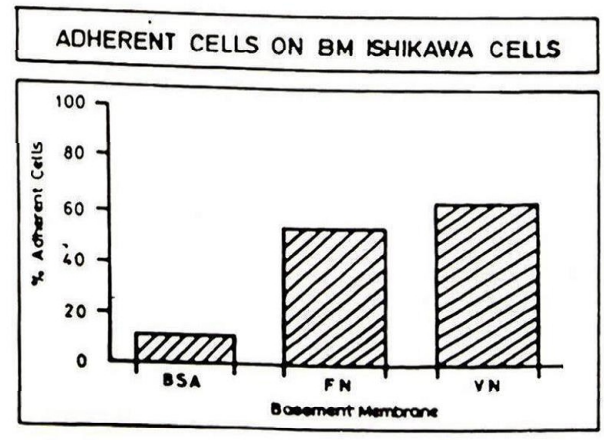

Fig. 3: Substrate adhesion assays in Ishikawa cells. Percentage adherent cells on various basement membranes—bovine serum albumin (BSA), fibronectin (FN), vitronectin (VN)

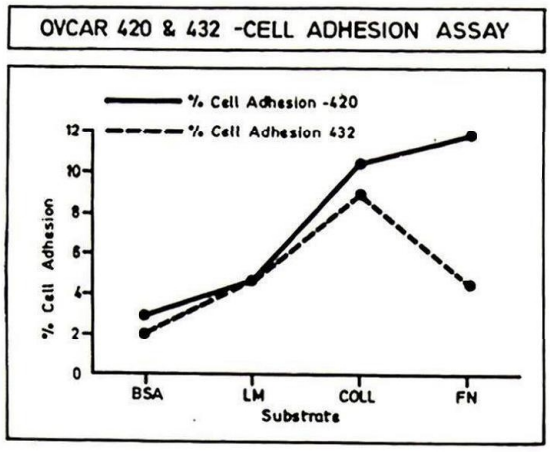

Fig. 4: Substrate adhesion: assays in ovarian cancer cell lives (Ovcar 420 + 432). Percentage adherent cells on substrates—bovine serum albumin (BSA), laminin (LM), collagen (Coll), fibronectin (FN)

Discussion

Enzymes are increasingly being used as markers in immune reactions with sensitivities similar to those obtained with isotopes [1]. The measurement of cell numbers using the ubiquitous lysosomal enzyme, hexosaminidase was first shown by Koponen *et al.* [2]. Since then researchers have demonstrated some interests in its applications. In the present study, the technique has been characterized and applied to quantitation of cell numbers and such common cellular assays as cell adhesions. Cell adhesions have been implicated in tumour growth, invasion, and metastasis. Their interactions in these regards with extracellular matrices and ligands have been amply demonstrated [3,4]. The choice of endometrial and ovarian cancer cell lines in demonstrating the value of the enzyme hexosaminidase in these assays is appropriate as it offers researchers another alternative to isotopes in evaluating and studying the behaviour of these cells in research.

The method described here readily lends itself to measurement of cell numbers, provided identical cell types are compared with a standard. Landegren [1] had previously shown that with an incubation time of 16 hours as few as 800 CTLL-2 cells were detected. The enzyme is quite stable and it resists freezing of cells for a number of days without any effect upon activity. Also, its activity is constant over time even when it is extended to 16 hours; however an ideal incubation period should be determined for each cell type. Serum contains a high level of hexosaminidase [2] and it is for this reason that the cells are washed repeatedly with Tyrodes Hepes/BSA buffer before the enzyme assay.

The adhesions of Ishikawa and Ovcar cells to ligands was analyzed with findings similar to previously reported results obtained by other methods [1,2]. As expected, the cells adhere least to bovine serum albumin (BSA). However, the experiments carried out in this study were essentially to demonstrate the value of the enzyme hexosaminidase. Landegren [1] has shown the value of this method as a sensitive and practical means of analyzing cell populations for the expression of surface antigens as well as for determining the ability of moni-antibodies to bind to a given cell type; besides the method is currently being used to screen hybridomas producing antibodies with specificity for cell surface antigens.

In conclusion, the technique of measuring cells via their content of hexosaminidase as shown in this study is applicable to a sizeable fraction of assays performed in immunology laboratories. The methods are rapid, sensitive, and inexpensive. Its non-radioactive nature is of tremendous advantage for its applicability in developing countries as it can easily be set up even in rural research centres. In addition many other assays involving benign and malignant cell quantitation assays could be adapted to similar measurement.

References

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