HISTOCHEMICAL DEMONSTRATION OF ACID BETA-D-GALACTOSIDASE ACTIVITY IN CULTURED HUMAN SKIN FIBROBLASTS AND ITS APPLICATION TO SINGLE CELL ANALYSES

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In 1970, Lojda reported the improved indigogenic method for β -D-galactosidase (β -gal). This method was applied to cultured skin fibroblasts from patients with three β -gal deficient disorders and from normal subjects. Cells from normal subjects showed the formation of insoluble blue indigo dye, whereas cells from the patients remained unstained. Clear-cut differences were observed in the intensity of the β -gal activities between cells from normal subjects and from the patients. This method was also applied to cultured amniotic fluid cells, and to the judgment of a genetic complementation study in restoration of β -gal activity.

Several methods have been proposed concerning the histochemical demonstration of acid β -gal activity in tissue *in vivo*, such as the indigogenic method (Lojda) (14), the azo indoxyl method (Gossrau) (6), the azocoupling method with 1-naphthyl- β -D-galactoside (Gossrau) (4), and the azocoupling method with naphthol AS-BI β -D-galactoside (Gossrau) (5). However, only a few publications have been concerned with the successful demonstration of acid β -gal activity in cultured human skin fibroblasts (7, 11, 12). We successfully employed the modified indigogenic method originally reported by Lojda (14) to demonstrate acid β -gal activity in cultured skin fibroblasts. This method was found very useful in diagnosing several hereditary disorders with β -gal deficiency. Cells from normal subjects showed the formation of insoluble blue indigo dye, whereas cells from the patients remained unstained. Clear-cut differences were observed in the intensity of β -gal activities between cells from normal subjects and from the patients. Since several different genetic metabolic disorders with β -gal deficiencies have been described (13, 16, 17), a better histochemical method for acid β -gal in cultured skin fibroblasts is necessary at present. The purpose of this paper is to describe the details of our histochemical method and to describe the application to single cell analyses in the genetic complementation study and in the prenatal diagnosis of β -gal deficient disorders.

MATERIALS AND METHODS

Cell Strains

Skin biopsies were obtained by the pinch technique and fibroblast culture was initiated and maintained in Eagle's minimum essential medium, supplemented with 10% fetal bovine serum (FBS, Flow Lab.), kanamycin and fungizone. Cell strains used in this study are summarized in Table 1.

For enzyme histochemistry, cells were placed in 6 cm plastic dishes containing 5 ml growth medium. In each plastic dish, one coverslip, 24 × 32 mm in size was placed in advance. After 24–48 hr, cells attached to coverslip were fixed *in situ* and processed for enzyme histochemistry.

Amniotic fluid cells suspected to have a β -gal deficiency were obtained by transabdominal amniocentesis at 15 weeks of gestation and the culture was initiated in 6 cm dish containing one coverslip with 2.5 ml amniotic fluid and 2.5 ml growth medium, supplemented with 20% FBS.

Cell Fusion

Equal numbers of cells were cocultivated in a 6 cm dish. The induction of cell fusion with polyethylene glycol (PEG) (mol. wt. 6,000–7,500, J. T. Baker Chemical Co.) was carried out according to the method of Davidson and Gerald (2) except that Eagle's minimum essential medium was used as medium. At 24–48 hr after fusion, cells were trypsinized and processed for biological enzyme assay and for

TABLE 1. *Lysosomal enzyme activities¹ in cultured skin fibroblasts*

Disorders	Strains	β -gal ⁵	β -hex ⁶	β -gluc ⁷	α -man ⁸	α -fuc ⁹	α -glu ¹⁰	β -glu ¹¹
ML-II ²	91	25	2706	74	22	3	91	98
	176	8	2178	44	10	3	84	n.d. ¹²
	177	21	1439	42	14	2	61	63
	186	35	1244	34	16	4	19	63
GM-1 ³	51	17	5067	248	76	n.d.	67	n.d.
	82	3	19648	168	365	48	180	101
	132	2	7500	133	213	85	25	23
	183	2	11818	114	98	207	30	158
ML-gal ⁴	20	40	13210	845	282	364	320	69
	108	48	6054	200	107	86	63	125
Normal	104	600	14400	189	129	138	89	71
	169	303	5700	225	155	32	81	151

¹ Enzyme activities are expressed as nmol of hydrolysed 4-methylumbelliferyl (4MU) substrate/mg protein/hour

² Mucopolipidosis II (I-cell disease)

³ GM₁-gangliosidosis

⁴ β -galactosidase deficient type mucopolipidosis

⁵ 4MU- β -galactosidase

⁶ 4MU- β -N-acetylglucosaminidase

⁷ 4MU- β -glucuronidase

⁸ 4MU- α -mannosidase

⁹ 4MU- α -fucosidase

¹⁰ 4MU- α -glucosidase

¹¹ 4MU- β -glucosidase

¹² Not determined

TABLE 2. *Composition of Lojda's medium*

5-bromo-4-chloro-3-indolyl- β -D-galactoside	3 mg
Dissolve in N,N-dimethylformamide	0.3 ml
0.1 M citric acid phosphate buffer, pH 5.0	7 ml
1.65% potassium ferricyanide	0.5 ml
2.11% potassium ferrocyanide	0.5 ml
Mix well and filter	about 8 ml

histochemistry. Control dishes not treated with PEG were also prepared.

Fixation

Prior to fixation, coverslips were rinsed with Hank's balanced salt solution to remove the serum components. Cells were then fixed in cold Lillie's neutral formalin for 30 min. After fixation, cells were rinsed twice in cold Holt's gum sucrose (9), and kept overnight at 4°C.

Enzyme Histochemistry

To demonstrate acid β -gal activity, cells were incubated for 5 hr at 37°C in Lojda's medium containing 5-bromo-4-chloro-3-indolyl- β -D-galactoside (Sigma Chemical Co.) as substrate (Table 2) (14). The same medium without the substrate was used for the blank experiment. Sodium chloride was added to the medium in a final concentration of 0.1 M, galactonolactone in a final concentration of 5×10^{-3} M, gluconolactone in a final concentration of 4×10^{-4} M.

Light Microscopy

After incubation, coverslips were rinsed twice with distilled water, counterstained with nuclear fast red for 10 min, then rinsed with distilled water, mounted with glycerine jelly and examined by light microscopy. The complementation was judged under a magnification of $\times 200$.

RESULTS

The cells from normal subjects showed a deposition of insoluble blue-green granules in the cytoplasm around the clear nucleus (Fig. 1). There was no background staining. On the other hand, the cells from mucopolipidosis II (ML-II), GM₁-gangliosidosis (GM-1) and β -gal deficient-type mucopolipidosis (ML-gal) were unable to hydrolyze the substrate. These cells remained unstained and clear throughout the incubation (Fig. 2). Cells from ML-II showed a scattered weak staining area. But clear-cut differences were observed in the intensity of the β -gal activity between normal and pathological subjects. The reproducibility of the reaction was excellent. The addition of sodium chloride in a final concentration of 0.1 M to the incubation medium resulted in a higher intensity of staining. The staining was completely inhibited by the addition of galactonolactone in a final concentration of 5×10^{-3} M. It was not influenced by the addition of gluconolactone in a final concentration of 4×10^{-4} M.

Cultured amniotic fluid cells suspected to have a β -gal deficiency showed no indigo staining (Fig. 3). Control cells showed a stronger reaction than cultured skin fibroblasts (Fig. 4).

FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

FIG. 5.

FIG. 6.

FIG. 1. Cells from normal subjects showing the deposition of insoluble indigo dye in the cytoplasm around the clear nucleus. $\times 200$

FIG. 2. Cells from the patient with mucopolipidosis II showing no indigo staining. $\times 200$

FIG. 3. Cultured amniotic fluid cells suspected of having a β -galactosidase deficiency showing no indigo staining. $\times 200$

FIG. 4. Cultured amniotic fluid cells from control fetus showing more strong indigo staining than cultured skin fibroblasts. $\times 200$

FIG. 5. Fusion of cell strains between GM₁-gangliosidosis and β -galactosidase deficient-type mucopolipidosis showed positive indigo staining in multinucleated cells. $\times 200$

FIG. 6. Fusion of cells among GM₁-gangliosidoses showed no indigo staining in multinucleated cells. $\times 200$

In the complementation studies, self-fusion of individual mutant or normal cells always gave staining results similar to those of the unfused parent cells. Fusions of cell strain from one type of β -gal deficiency and from the different type of β -gal deficiency always produced positive indigo staining in multinucleated cells (Fig. 5). Mononucleate cells always remained unstained and clear. Moreover, fusions among cells from the same type of β -gal deficiency produced no indigo staining (Fig. 6).

DISCUSSION

In general, the histochemical demonstration of enzyme activity in cultured skin fibroblasts is more troublesome than in tissue *in vivo*. Chang *et al.* (1, 15) speculated that the physical properties of the cell surface of monolayer cells were quite different from those of cells in tissue *in vivo*. Gravel *et al.* (7) described a histochemical method using the same substrate as ours. However, their method required overnight incubation and there was a possibility of background staining. Incubation with substrate solution seemed too early because it was started after 5 min of fixation by their method. The reproducibility of the reaction might be inhibited by the possible contamination of formalin. In our method, the washing with Holt's gum sucrose after fixation can shorten the incubation period in spite of long fixation, that is, to 30 min. The reproducibility of the reaction is excellent in our method.

The usefulness of our method has been demonstrated in regard to diagnosis, genetic complementation and prenatal diagnosis of β -gal deficient disorders. In our method, clear-cut differences have been observed in the intensity of the β -gal activity between cultured skin fibroblasts from normal subjects and from those with β -gal deficient disorders. And the intensity and pattern of the staining by only 5 hr incubation enable the differentiation of mutant cells from normal ones. These results and the addition of sodium chloride, galactonolactone and gluconolactone clearly show the specificity of β -gal staining (8, 14).

In the genetic complementation study, the judgment of complementation was very easy and the results were compatible with the previous report (19). Fusion frequencies are not always sufficient to detect complementation and dilution of heterokaryons by parental cells is problematic. Thus the single cell analyses were devised (3, 10) and we previously reported a simple method (18). However, skillful technique and expensive tools are necessary for such analyses. In the present study, the histochemical method for β -gal activity has clearly been shown to be a useful and reliable qualitative method for single cell analyses.

In prenatal diagnosis, cultured amniotic fluid cells suspected of having a β -gal deficiency showed no indigo staining. Further enzymatic study confirmed this fetus as β -gal deficiency (Table 3). Termination of pregnancy was done at 20 weeks of gestation. Pathological studies of fetal tissues confirmed the diagnosis of storage disorder. Further studies of fetal tissues are now under way for the definitive diagnosis. Kaback *et al.* (11) reported a case of prenatal diagnosis of GM₁-gangliosidosis, type 1. They used the histochemical technique in conjunction with biological assay for β -gal activity, but the details of the histochemical method were not given. Large numbers of amniotic fluid cells are necessary for classical biochemical analysis. In most instances, 4–6 weeks of cultivation are required before biochemical

TABLE 3. *Lysosomal enzyme activities¹ in cultured amniotic fluid cells*

Enzymes \ Pregnancies	At risk	Control 1	Control 2
4MU ² - β -galactosidase	5	927	875
4MU- β -N-acetylglucosaminidase	10752	10212	10453
4MU- β -glucuronidase	240	354	285
4MU- α -mannosidase	246	346	183
4MU- α -fucosidase	1068	1681	839
4MU- α -glucosidase	n.d. ³	92	83
4MU- β -glucosidase	196	120	172

¹ Enzyme activities are expressed as nmol of hydrolysed substrate/mg protein/hour.

² 4-methylumbelliferyl

³ Not determined

studies can be performed for the prenatal diagnosis of metabolic disorders. Our method requires only a few viable amniotic fluid cells. Thus a shortening of the culture period is possible and in this case, the histochemical method was performed only after one week from the initiation of amniotic fluid cell culture. Our method will provide a useful addition to the methods available for prenatal diagnosis of β -gal deficient disorders.

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