CULTURE CONDITIONS FOUND TO MINIMIZE FALSE POSITIVE DIAGNOSIS OF LYSOSSMAL STORAGE DISORDERS

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SUMMARY

The effect of culture conditions on the ultrastructure and enzyme activities of cultured skin fibroblast cells relevant to the diagnosis of lysosomal storage disorders are reported. The parameters examined were: pH of the culture media, type of media, increasing cell passage, and day of harvest. Ultrastructural changes were defined in terms of the number of lysosome-like inclusion bodies per cell according to a method devised in our laboratory and proven reliable in the detection of affected individuals. Our biochemical results included determination of enzyme activities of β-hexosaminidase, α-mannosidase, β-glucuronidase-lysosomal enzymes, arylsulfatase C, a microsomal marker, and 5′ nucleotidase, a plasma membrane marker. Our results indicate that the cellular ultrastructure is more sensitive than enzyme activity to changes in culture conditions. The resulting ultrastructural "artifacts" observed under certain conditions were severe enough to result in a mistaken diagnosis. Due to certain difficulties we had previously encountered in heterozygote cultures (for lysosomal storage disorders) of amniotic cells, we decided to examine heterozygote cultures of skin fibroblasts. From these (preliminary) studies it seems that an elevation in the pH over the physiologic levels in the culture media may help to define between normal individuals and affected heterozygotes. On the basis of our results, we recommend that to minimize false positive ultrastructural results for the diagnosis of lysosomal storage disorders, cultures be grown in minimal essential medium, the pH of the medium carefully monitored to remain below 7.4, examining the cultures not later than cell Passage 8 and no later than Day 10 after subculture.

Key words: skin fibroblasts; ultrastructurc; cell culture; lysosomal storage.

INTRODUCTION

Transmission electron microscopy (TEM), has been recognized for several years as an important tool for the diagnosis of lysosomal storage disorders. It is used either for examination of direct biopsies (skin, conjunctiva, liver, brain, etc.) or, more often, for examination of cultured cells, mainly cultured skin fibroblasts. However, a major drawback in the use of the cultured cells is that certain culture conditions may introduce nonspecific ultrastructural and enzymatic changes which may result in a false diagnosis. A number of parameters that have been shown to affect enzyme activities, as well as ultrastructural findings in skin fibroblasts, include pH of the media, frequency of media change, day of harvest, and type of media or buffer used (5,13,22).

In view of these findings, and because some lysosomal storage disorders are diagnosed on the basis of TEM findings alone (in addition to the typical clinical picture), we found it necessary to methodically determine the optimal conditions for growing skin fibroblasts cultures for the purpose of diagnosis of lysosomal storage disorders. We examined the effect of pH of the culture medium, increasing cell passage, and day of harvest and tried to find the correlation between the cellular ultrastructure and enzymatic activities of cultured skin fibroblasts from people who are homozygous nonaffect- ed. We chose for enzymatic studies β-glucuronidase, β-hexosaminidase, and α-mannosidase as lysosomal en-

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MATERIALS AND METHODS

Skin fibroblast cultures. Skin biopises were obtained from children and adults not known to be affected with any storage disorder, and were prepared into primary cultures. The biopsies, which included epidermis, dermis, and subcutaneous tissue were minced and placed in a 25-cm² culture flask (Nunc) containing Ham's F10 nutrient medium supplemented with 20% fetal bovine
serum (FBS), 2 mM L-glutamine, 50 U penicillin, and 50 µg streptomycin per milliliter (GIBCO, Grand Island NY). Cells were grown in culture flasks, maintained at 37°C in 95% O₂:5% CO₂, and were refed every 4 d. After reaching confluency, the cultures were split (this was considered Passage 1) and were fed with Eagle's minimum essential medium (MEM) supplemented with 10% FBS and, unless specified otherwise, the cultures were refed every 4 d, as well as the day before and the day after subculture. All cultures were examined ultrastructurally and biochemically. In addition, we received a number of cell lines from the Dept. of Human Genetics, Hadassah University Hospital, which were similarly prepared, but were frozen at various cell passages.

We initially studied the effect of different culture media. Replicate aliquots of each cell line were inoculated into two flasks. One flask was fed MEM and the second flask was fed Ham's F10. Each flask was subcultured, and the cells were removed for enzymatic and ultrastructural examination.

The effect of pH of the medium on cellular ultrastructure and enzymatic activities was examined. In an initial experiment, replicate aliquots of each cell line were inoculated into three flasks. Each flask was fed with MEM buffered with sodium bicarbonate, at pH 6.6, 7.2, and 8.0. In spite of changing the medium daily, wide variation occurred in the final pH values. The experiment was later repeated using MEM with the addition of 30 mM tricine buffer.

The experiment was repeated using cells from known heterozygotes and homozygotes for Neimann-Pick and Mucolipidosis IV. The cultures were grown in MEM at different pH levels with the addition of tricine buffer.

To study the effect of increasing cell passage on skin fibroblasts, the cultures were subcultured up to Passage 20 and all cultures were examined biochemically at Passages, 3, 5, 7, and 10 and ultrastructurally till Passage 20. Cultures were grown in MEM and were examined on Day 5 after subculture.

To compare the effect of day of harvest on cellular ultrastructure and enzymatic activities, each cell line was divided equally into three flasks. The initial inoculate was sufficient large to ensure that each flask reached confluency before being harvested. Cultures were fed with MEM and the cells harvested on Days 5, 10, and 14 after subculture.

Transmission electron microscopy (TEM). Cultured skin fibroblasts were removed by trypsinization, washed twice in cold, normal saline, and the resulting pellet divided into two halves. For TEM the cells were fixed in 2.5% gluteraldehyde in 0.1 M buffer cacodylate, rinsed in buffer cacodylate, postfixed in 2% osmium tetroxide in 0.1 M buffer cacodylate with 5% sucrose, dehydrated in a series of graded alcohols, and embedded in araldite in Beem capsules. Thin sections were stained with uranyl acetate and lead citrate and examined by a Philips 300 electron microscope.

Only cell profiles sectioned at the level of the nucleus were studied. Cells were considered to be normal if they contained less than five single membrane bounded vesicles (MBV) per cell. When 5 to 10 inclusions per cell were found, the cell was considered to be “slightly affected” and “severely affected” when more than 10 inclusions per cell were present. These inclusions contained either lamellar, granular, or amorphous material or, in some cases, were empty of material. In most cases 80 to 100 profiles per culture were examined from at least two separate blocks (18).

Biochemical examination. The cells in the remaining pellet were resuspended in double distilled water and the cells lysed by repeated freeze thawing five times. Total protein was determined by the Lowry method (15).

Total β-hexosaminidase activity was measured using 2 mM 4-methylumbelliferone-2-deoxy-β-D-glucopyranoside (Koch-Light Lab) as substrate in 0.1 M buffer citrate, pH 4.5, as previously described (3).

β-Glucuronidase activity was measured by 2 mM 4-methylumbelliferone-β-D-glucuronide tridydrate in 0.1 sodium acetate buffer, pH 5.0 (3).

α-Mannosidase activity was measured using 2 mM 4-methylumbelliferone-α-D-mannopyranoside as substrate in 0.1 M citrate phosphate buffer, pH 4.5 (20).

Arylsulfatase C, a microsomal enzyme, was measured in a mixture containing 2 mM 4-methylumbelliferone-sulfate in 0.1 M buffer phosphate, pH 8.0 (17).

The plasma membrane marker, 5’ nucleotidase was measured according to Aronson and Touster (2) by the

![Fig. 1. a, TEM photomicrograph of skin fibroblast grown in MEM, taken from normal individual. X15 000. b, skin fibroblast grown in F10, taken from normal individual. Cell seems severely affected with increase in lysosome-like membrane-bounded vesicles (arrows). X32 000. Stained in lead citrate and uranyl acetate.](image-url)