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L-GLUTAMATE TOXICITY IN HUNTINGTON'S DISEASE FIBROBLASTS

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SUMMARY: Brain degeneration in Huntington's Disease is thought to occur primarily in the regions of high L-glutamate concentrations. Huntington's Disease fibroblast cultures have a sensitivity to these high concentrations of L-glutamate. These cells show degeneration and loss of viability, within 12 hrs, following treatment with 30 mM L-glutamate. This effect appears to be specific for L-glutamate, can be prevented by glutamine, and is not observed in matched control cultures. The observed glutamate sensitivity may serve as a biochemical genetic marker and the excessive effect of glutamate on neuronal membranes could initiate the characteristic neuronal degeneration observed in Huntington's Disease brains.

INTRODUCTION

Glutamic acid decarboxylase activity and γ-aminobutyric acid pools are increased in Huntington's Disease fibroblasts in culture (1). Altered glutamine utilization in membrane synthesis has been observed in similar cells (2,3) and additional evidence for altered membranes in HD cells has been obtained from fibroblasts (4,5), erythrocytes (6) and brain slices (7). One consequence of the membrane alteration in HD cells seems to be an increase in the rate of uptake for L-glutamate (8). Few compounds are increased in HD brains. Increased γ-hydroxy butyrate (9), glutamate dehydrogenase (10) and protein P50 (11) have been observed. Total amino acid analysis of the putamen and caudate nucleus showed a general decrease in cellular amino acids, except for glutamate, which was present either in higher amounts (12) or with no significant loss in HD substania nigra (13). Values for glutamate in HD cortical biopsies were only slightly reduced (14).

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Abbreviation: HD - Huntington's Disease.

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The changes observed in the HD tissues outside the brain probably reflect a subtle structural alteration in the cell. This alteration is not expressed in vivo, because the extracellular environment is very different from that in the brain. Glutamate is the most abundant free amino acid in the brain where concentrations range from 8 to 20 mM (15,16). The concentrations of glutamate in other organs are approximately one-third the brain values. Glutamate outside the brain is relatively non-toxic (17,18). However, when high concentrations of glutamate are injected into specific portions of the brain glutamate can cause severe neuronal necrosis (19,20,21). This tissue degeneration is similar, but not identical, to that observed in HD brains. In an effort to determine whether glutamate could be involved in HD brain cell degeneration, HD fibroblasts were exposed to the high L-glutamate concentrations found in the brain. The HD cell cultures showed loss of cell viability when treated with 20-40 mM L-glutamate. Control cultures did not respond in the same way and the HD cell death was specific for L-glutamate.

MATERIALS AND METHODS

Cell Cultures: HD fibroblast cultures were obtained from the Camden Mutant Cell Repository (Camden, NJ) and from confirmed HD patients in the area. The HD cells obtained from the Camden Mutant Cell Repository were GM numbers 2165, 1650, 1085, 1170, 1169, 1061, and 305. Twelve additional HD cell cultures have been cultured from skin biopsies. The age of the HD patients represented are 28 yrs to 65 yrs. Age and sex matched control cultures were obtained locally and from the Camden Mutant Cell Repository (GM 43 and 2185). All cells were grown routinely in Dulbecco's Modified Eagle's Medium (MED No. 72214, Gibco, Grand Island, NY) containing 4.5 g glucose per liter, 4 mM glutamine, 100 mg per liter sodium pyruvate and 20% fetal calf serum, heat inactivated, virus tested and Mycoplasma screened. Cells were grown as monolayers in plastic tissue culture flasks at 36.5°C in a humidified atmosphere of 6% CO_2 - 94% filtered air. In experiments requiring varying amounts of particular amino acids, such as glutamate and glutamine, the medium was prepared accordingly. All cultures were routinely checked for Mycoplasma contamination using the Hoescht fluorescent staining technique and the culture technique, in 95% N_2: 5% CO_2 (Flow Laboratories, McLean, VA).

Cell viability was determined by the trypan blue and alcian blue dye exclusion tests in fixed and unfixed cells (22,23). Dye exclusion was determined in situ and after cell detachment, with comparable results. Counting of cells in situ was done with the aid of a microscope eyepiece having an internal grid. Detached cells were counted and visually differentiated using an hemocytometer. Some cultures also were examined for [methyl-^{3}H]-thymidine incorporation into DNA, [^{3}H]-leucine incorporation into proteins, and cell enumeration with an electronic particle counter.

Toxicity Studies: The toxicity studies on the HD fibroblasts used cultures twenty four hours after plating at 1.5 x 10^4 cells/cm^2 in 8 cm^2 plastic tissue

culture dishes. Cells were plated in medium containing 20% fetal calf serum. After 24 hours the medium was removed and replaced with fresh medium, plus serum, containing the desired concentrations of glutamate, glutamine or other compounds to be tested. Treated cells were incubated at 36.5°C for 36 hours and monitored microscopically every 12 hours for cell death. All cultures were set up in quadruplicate so viability testing could be done by at least two methods. Visual monitoring of the cultures was expressed as per cent cell death (detached, floating and degenerating cells) compared to untreated controls. This is a subjective, but rapid, screening technique. Each culture, after 36 hrs, was tested for viability.

Several compounds were tested individually for toxicity to the HD and control cells. These were added to the medium from 0.5 M or 1.0 M stock solutions in serum free medium, pH 7.2. Some of the compounds were also examined for an inhibitory effect upon glutamate toxicity. In these experiments the test compound (30 mM) was added to the cells in the presence of 30 mM glutamate. Control cultures, the non-HD cells and the HD cells not treated with glutamate, included standard medium and medium with sodium chloride added to the total concentration of test compounds in the cultures. Amino acids were added as sodium salts, pH 7.2.

Uptake Studies: Intracellular accumulation of L- [U-^{14}C] - glutamate by HD and control cells was measured in the presence of increasing exogenous glutamine. Uptake was examined in the presence of 30 mM L-glutamate, 0.1μCi/ml L- [U-^{14}C]-glutamic acid (270 mCi/mmol, Amersham, Arlington Heights, IL). Cells were plated in multi-well plates, 2 cm^2/well, and grown to a cell density of 3 x 10^4 cells/cm^2. The medium was replaced with serum free medium or a buffered saline solution containing the L-glutamate and glutamine. The cells were incubated at 37°C for 15 min, rinsed three times with ice cold Tris-HCl saline, pH 7.2, air-dried and solubilized in 0.2 M sodium hydroxide. One half of each sample was retained for protein determinations (24) and one half was neutralized for determination of radioactivity. Experimental values were determined in quadruplicate for each culture.

RESULTS AND DISCUSSION

The effects of glutamate, up to 50 mM, were determined on nine HD fibroblast cell cultures. Non-HD cultures, matched for sex, age, and passage, were tested in parallel. Experiments testing the effects of several glutamine concentrations were performed using glutamate-free media. Control experiments included observations of growth in complete medium and medium supplemented with NaCl at concentrations equal to glutamate. All HD cell cultures showed characteristic cell death and degeneration within 16 hrs following treatment with glutamate in excess of 20 mM, Figure 1. HD cells grown in 10 mM glutamate had viability decreased by only 20%. Glutamate at 30 mM and 50 mM was extremely toxic to HD cultures 12 hrs after treatment. Cell death was accompanied by fragmentation rather than simple detachment and rounding up from the growth surface. Cell

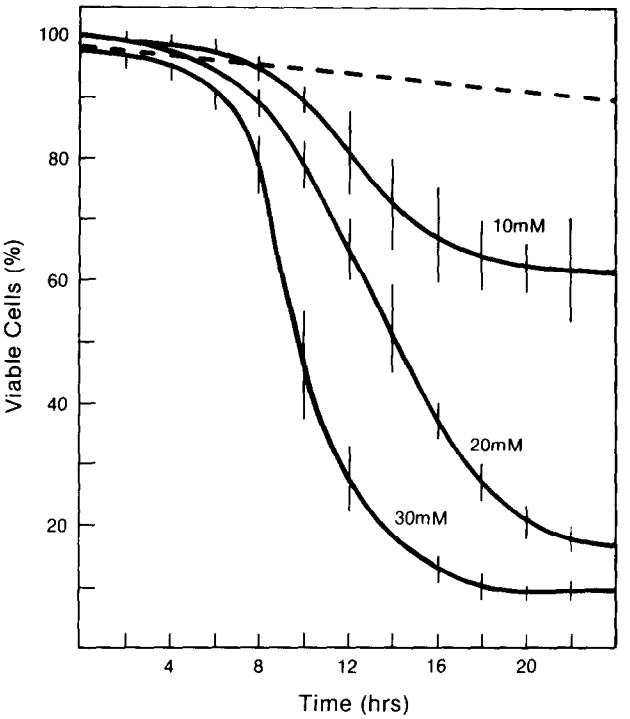

Figure 1. Viability of Huntington's Disease and control fibroblasts treated with L-glutamate. Solid lines represent Huntington's Disease cells treated with three concentrations of L-glutamate, 10 mM, 20 mM and 30 mM. The broken line is a composite line representing control cells treated with L-glutamate at the same L-glutamate concentrations and both cell types treated with 30 mM D-glutamate, glutamine, Y-aminobutyric acid, aspartate, cysteic acid, asparagine, proline, leucine, ornithine, sodium chloride or kainic acid. Cell viability was determined by dye exclusion (See Materials and Methods). Several hundred cells were scored for each time point. Cells from nine patients with Huntington's Disease and nine age and sex matched controls were examined. Vertical lines represent the standard deviation for each value. The abscissa is time following addition of test compound to the cell culture medium. Cells were plated and tested as described in Materials and Methods. The p values for the differences from control cells for HD cells in 10 mM, 20 mM and 30 mM L-glutamate are $p=0.15$, $p < 0.002$, $p < 0.001$, respectively.

death and degeneration were not observed in any of the matched control cell cultures grown in 30 mM glutamate.

HD cells did not die in the presence of 30 mM D-glutamate, pyroglutamate, γ-aminobutyric acid, α-ketoglutarate, aspartic acid, cysteic acid, proline, glutamine, asparagine, ornithine, sodium chloride, potassium chloride or ammonium chloride. Kainic acid, 30 mM, was also non-toxic to all cells tested. Glucosamine, 10 mM, was toxic to both control and HD cells. At high concentrations of the above compounds generation times were increased and accumulation of intra-

Table 1. Effect of Several Compounds On L-Glutamate Toxicity To HD Fibroblasts

	% Viable Cells (± standard deviation)	
	HD	CONTROL
No Treatment	> 98 (0.6)	> 98 (0.7)
L-Glutamate (30 mM)	6 (1.7)	91 (2.1)
+ Cysteic Acid	11 (2.3)	75 (6.4)
+ GABA	13 (1.8)	81 (5.3)
+ Glucosamine	< 1 (0.1)	< 1 (0.1)
+ D-Glutamate	42 (8.6)	74 (8.3)
+ Glutamine	76 (4.8)	91 (2.2)
+ Kainic Acid	5 (1.7)	93 (0.8)
+ Ornithine	10 (2.1)	86 (1.7)

Several compounds were tested for prevention of L-glutamate toxicity on fibroblast cultures obtained from control subjects and patients with Huntington's Disease. Cells were treated for 24 hrs in complete growth medium with 30 mM glutamate plus 30 mM test compound. Results are expressed as percent of viable cells as determined by vital staining with trypan blue and alcian blue. Values are averages from a minimum of ten determinations. The difference between control and HD cells treated with 30 mM L-glutamate has a $p < 0.001$.

cellular granules was noticeable. Both observations normally would be expected under these adverse growth conditions.

When HD cells were treated with toxic concentrations of glutamate, cell death could be prevented by simultaneous addition of equimolar glutamine. These observations correlate with the protective effects of glutamine reported for kainic acid treated rat brains (29,30). The possible protective effects of other compounds related to glutamate were examined by incubating cells in 30 mM glutamate plus 30 mM test compound, see Table 1. GABA, D-glutamate, ornithine, cysteic acid, kainic acid and glucosamine did not block the glutamate- induced cell death. Kainic acid, which has a potentiating effect on glutamate induced neuronal degeneration (31,32), did not potentiate the glutamate effect in the

fibroblasts. HD and control cells were treated with 10 mM glutamate plus 5 mM increments of kainic acid, up to 30 mM kainic acid, and no cell death was observed.

The toxic effects of glutamate were observed with early and late passage cells. Age of onset of HD symptoms and the age of the patient at time of biopsy did not seem to affect the study, although a larger population needs to be examined prior to confirming this.

Glutamate toxicity, similar to that observed in the HD fibroblasts, has been shown by several laboratories to occur in the brains of mice and rats. Selective neuronal degeneration in the mouse and rat brains was also obtained by lower concentrations of injected kainic acid, a glutamate analog (19,21,33,34). When applied to HD or control fibroblasts, kainic acid did not cause cell death. Since kainic acid is thought to act as an excitotoxin which mediates a glutamate induced cell death in the rodent brains, it is not unexpected that kainic acid would be ineffective on fibroblasts. HD cell degeneration, caused by glutamate, was prevented by simultaneous addition of equimolar glutamine. Short-term uptake studies revealed that 10 mM glutamine was capable of reducing glutamate incorporation by 60%. The mechanism for glutamine protection is unclear, although it may interfere with glutamate binding to the cell. Glutamate uptake, in brain tissue, is not inhibited by glutamine, GABA or glutarate, provided the external glutamate concentration is 1 mM. However, when the extracellular concentration of glutamate is 10 mM there is an approximately 17% inhibition of glutamate uptake by glutamine (25). This finding is similar to our fibroblast studies. In C-6 glioma cells, the presence of glutamine in the growth medium is essential for maintaining normal intracellular concentrations of glutamate, aspartate and glutamine (26). When glutamine was limited in the C-6 cells, synthesis of glutamate was reduced 40% and glutamine was no longer synthesized. This decrease resulted in a significant increase of the glutamate:glutamine ratio from 1.6 to 26. The inability of HD cells to maintain a proper balance between glutamate and glutamine may ultimately result in cell death. Preliminary

results from experiments designed to measure intracellular concentrations of glutamate and glutamine in HD cells during exposure to toxic and nontoxic amounts of glutamate substantiate this concept (27).

Events similar to those observed in the animal models for HD occur naturally and specifically in HD fibroblasts. The toxicity and subsequent cell degeneration apparently are caused by concentrations of the endogenous brain compound, glutamate, which are only slightly in excess of normal values. Toxicity can be retarded and prevented by increasing the levels of a primary glutamate precursor, glutamine. These results mirror a clinical study (28) reporting that high amounts of dietary glutamine given to HD patients caused an increase in GABA in the cerebrospinal fluid with concomitant improvement of choreic movements.

Our results provide evidence pertinent to both the primary membrane defect and the cause of cell degeneration in the HD brain; namely, an excessive effect of glutamate on neurons that may have defective membrane functions.

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