GLUCOSAMINE DEPENDENCE OF HUNTINGTON'S CHOREA FIBROBLASTS IN CULTURE

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SUMMARY: Huntington's chorea is an autosomal dominant disease of the nervous system. Fibroblasts of one such case obtained from the Genetic Mutant Repository have a normal growth rate when compared with an age, sex and passage number matched human fibroblast cell line obtained from the same source. However, when the culture medium is depleted of nutrients and non-essential amino-acids added either individually or in combination, the Huntington's chorea fibroblasts show a dependence for glucosamine in the culture medium for cell survival and replicative capacity. Glutamine cannot be used in place of glucosamine. In fact, there is a further increment of cell morphology and number deterioration by Huntington's chorea but not normal fibroblasts when glutamine is added to depleted cultures.

INTRODUCTION

Huntington's chorea is a devastating hereditary disorder of the central nervous system characterized by the appearance in adult life of progressive intellectual, emotional deterioration and abnormal movements of the body musculature. (1) The genetic defect that results in the abnormal functioning of neural networks in the brain is unknown. However, the phenotype is indicative of an abnormality in neural irritability and perhaps of an abnormality in the neuronal cytoplasmic membrane. Since Huntington's chorea is an autosomal dominant disease the phenotype may be expressed in other cell populations such as secondary skin fibroblasts in culture.

The gene dosage principle would predict that the cells of the heterozygous Huntington's chorea patient would have fifty percent of the normal level of a given enzyme or structural protein. This theoretical consideration would predict that if Huntington's chorea fibroblasts are found to express a nutritional auxotrophy it should be possible to elicit the condition by depleting the culture media of critical metabolites. The following strategy was used to test the hypothesis. Cells were allowed to grow to early confluence, from then on the culture media was unchanged to deplete metabolites that may be in critical supply

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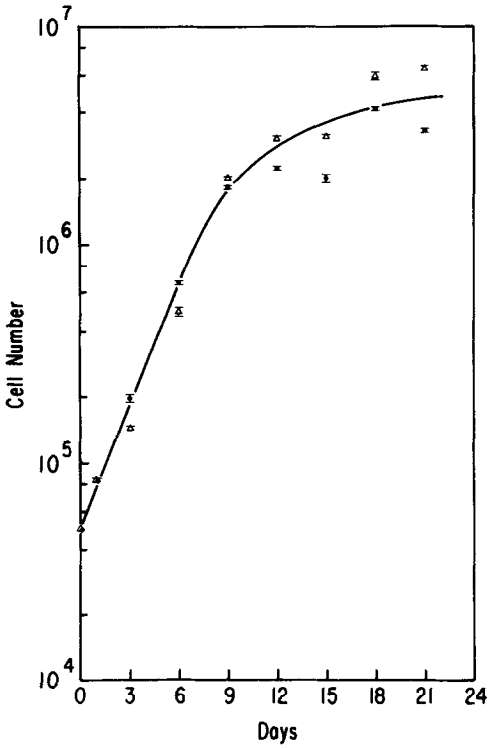


Figure 1.

Growth curves for Huntington's chorea and control fibroblasts.

Logarithmically growing cells were plated at a density of 50,000 cells per 25 ml flask in 5 ml of MEM with 20% dFCS. Fresh medium was added every third day. Cells were trypsinized and an aliquot counted in a ZBI Coulter counter. Each point represents the mean ± SEM of the cell number of five flasks.
△ = GM-124 normal cells; ● = GM-305 Huntington's chorea fibroblasts

to such cells. This condition was followed by selectively supplementing and replenishing the culture medium one component at a time with nutrients of interest. If a metabolic block exists, bypassing the block by adding the product distal to the block should allow the cells to survive and have normal reproductive capability.

Our results, as presented in this report, show that Huntington's chorea fibroblasts require glucosamine for survival and multiplication when culture media is depleted of nutrients, and show an adverse affect in morphology and cell number when glutamine is added in place of glucosamine.

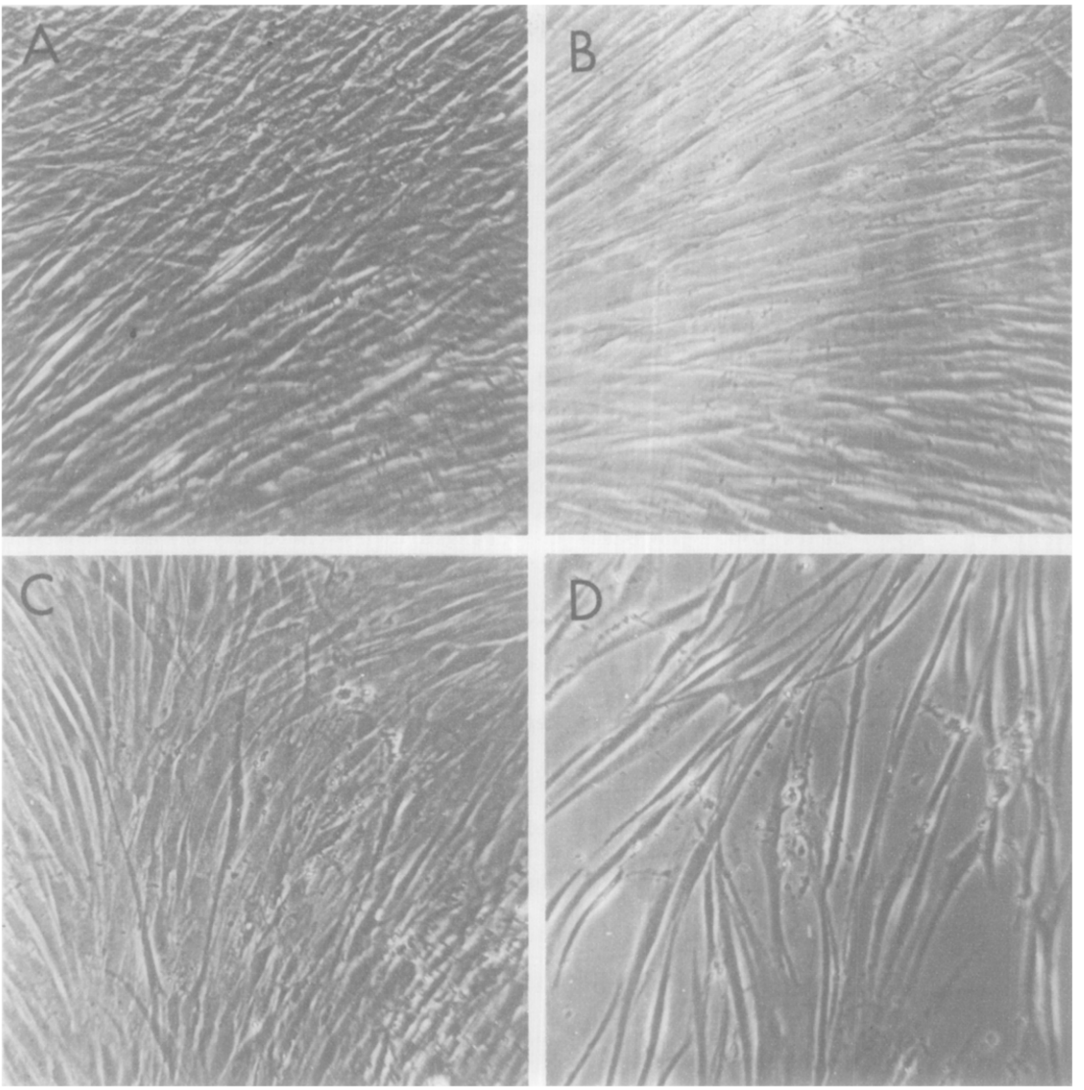


Figure 2a, 2b.

Confluent culture fibroblasts from normal and Huntington's chorea.

Cells were plated and grown identically as in Figure 1. Phase contrast photomicrographs were taken on day fourteen.
A = GM-124 normal; B = GM-305 Huntington's chorea

Figure 2c, 2d.

The appearance of normal and Huntington's chorea fibroblasts in nutrient depleted culture media.

Logarithmically growing cells were plated at a density of 50,000 cells per 25 ml flask in 5 ml MEM with 20% dFCS. Fresh medium was added on day 3. From then on the media was unchanged. The phase contrast photographs were taken 21 days after plating. There was no change in the pH of the medium.
C = GM-124 normal; D = GM-305 Huntington's chorea
Magnification of original photomicrograph x 450

METHODS AND MATERIALS

Huntington's chorea skin fibroblasts (GM-305) from a 56 year old white female were obtained from the Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, New Jersey. Control fibroblasts (GM-124) from a 59 year old white normal female was also obtained from the same source. Culture media and dialyzed sera were obtained from GIBCO. Amino acids and glucosamine were obtained from Calbiochem.

Minimal Essential Medium (MEM) with Earle's salts and dialyzed 20% fetal calf serum (dFCS) were routinely used for culture media. No antibiotics or additional components were added to the culture media.

Logarithmically growing cells were treated for 5 minutes by trypsin (0.04% in Puck's saline A with 0.02% EDTA). Cells were counted in a Coulter counter. Automatic pipettes were used for replica plating. Fifty thousand cells were routinely plated in 25 ml Falcon plastic flasks and 5 ml of minimal essential medium (MEM) with Earle's salts and 20% dialyzed fetal calf serum (dFCS) was added to each plate. Quintuplicate flasks were used for each category of nutritional supplementation. The media was changed every third day unless otherwise indicated. Phase contrast microscopy was used to photograph culture flasks.

RESULTS

The growth characteristics and morphology of GM-305 and GM-124 are identical when 20% dFCS and MEM are added to a series of quantitatively plated cells and the culture medium changed every three days. (Fig. 1, 2a, 2b)

After the fourth day of plating of cells the changing of the culture media was discontinued. Marked morphological changes of GM-305 fibroblasts were observed, compared to GM-124 normal fibroblasts. These changes are characterized by thinning, elongation and the appearance of sparcity and are first observed approximately 10 days after the last change in media. (Fig. 2c, 2d)

Supplementation of depleted cultures on the seventh day and every three days thereafter with individual non-essential amino acids either singly or in combination does not result in any reversal of the morphological changes depicted in Fig. 2d for GM-305. The addition of glutamine (Gln) to depleted cultures results in a further increment of quantitative and morphological deterioration of GM-305. (Fig. 3a, 3b) However, the addition of glucosamine (GlcN) or N-acetylglucosamine (GlcNAc) (not shown) dramatically restores the fibroblasts of GM-305 to normal morphology and survival. (Fig. 3c, 3d)

Depleted cultures that were supplemented with various amino acids or glucosamine for a period of three weeks were dissociated and counted. Fifty

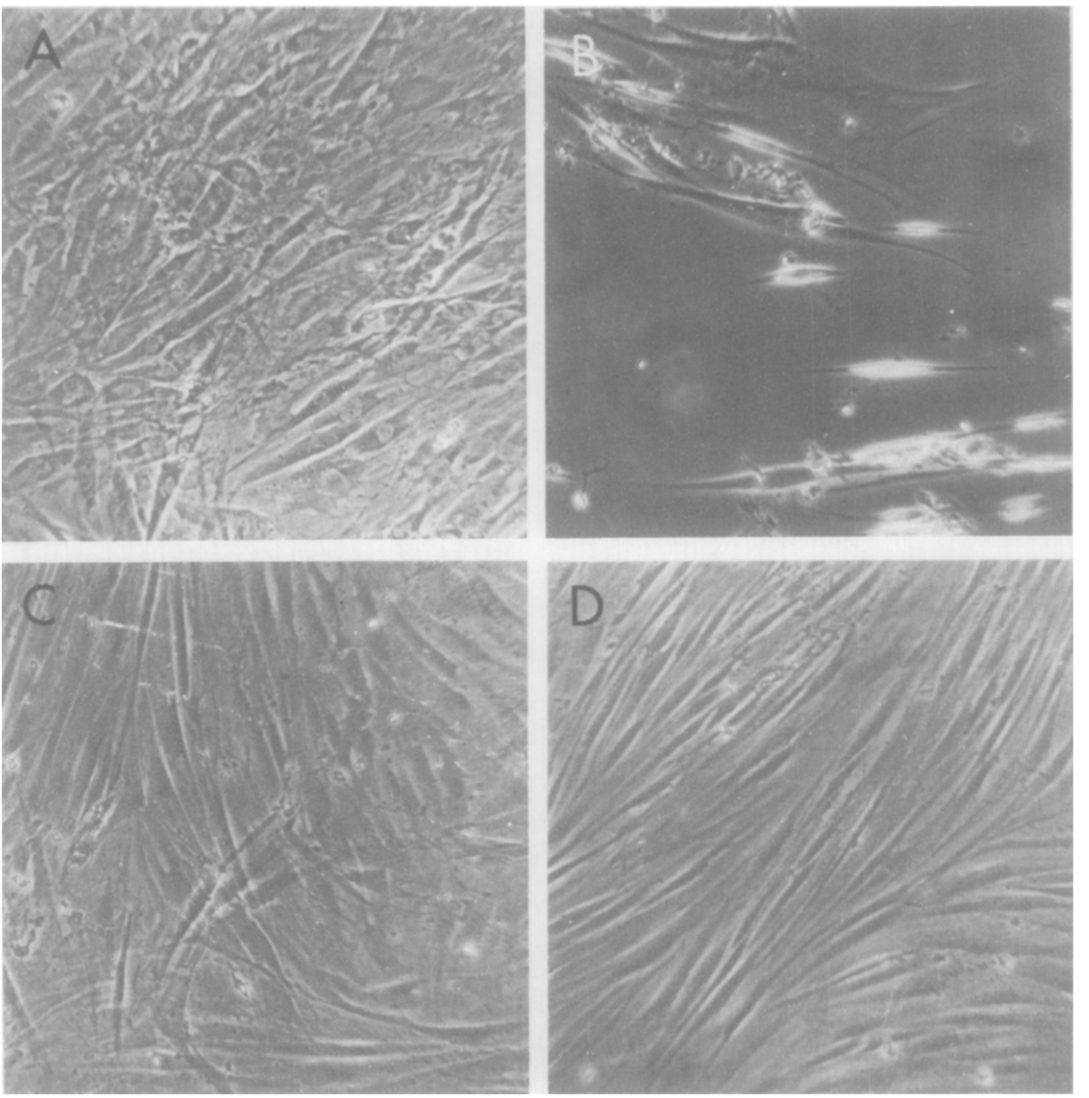
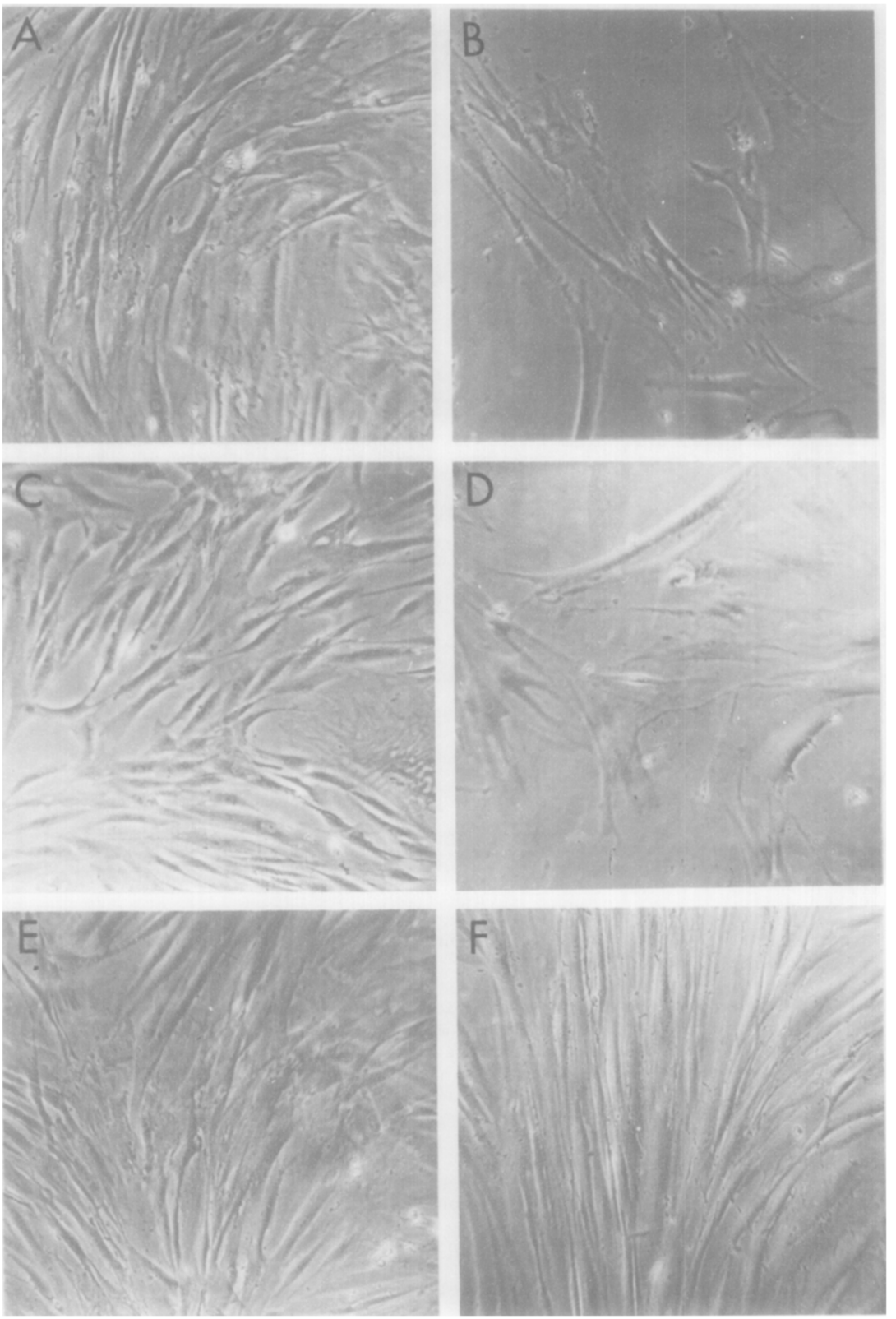


Figure 3.

Huntington's chorea fibroblasts when glutamine or glucosamine is supplemented to nutrient depleted cultures.

Cells were plated and grown identically as in Figure 2 except that on day 7 and every 3 days thereafter 1 mM glutamine (A and B) or glucosamine (C and D) in 0.5 ml volume was supplemented to the culture medium. There was no change in pH of the culture medium during this phase. The phase contrast photographs were taken 21 days after plating. Magnification of original photomicrograph x 450.

thousand cells from each experimental group were replated in normal 20% dFCS and MEM medium and allowed to grow for two weeks at which time cell morphology and growth rate as judged by the number of cells in each category were re-evaluated.


A
B
C
D
E
F

(Fig. 4a-4f) It is obvious that GM-305 cells do well only when supplemented with glucosamine. While normal GM-124 cells do well with supplementation with glutamine, glycine + asparagine + glutamine, or glucosamine. Additionally GM-305 cells that were not supplemented with glucosamine have grossly distorted morphology and a marked decrease in number of cells.

DISCUSSION

There are two reports in the literature on the growth characteristics of Huntington's chorea fibroblasts in culture. The first by Menkes and Stein 1973 (2) have shown a reduced replicative life span of the fibroblasts from Huntington's chorea patients. The second was reported by Goetz et al. 1976 (3) who could not show any difference in replicative life span of Huntington's chorea fibroblasts but observed a normal initial growth rate with a significantly higher maximal density at the stationary phase of growth. We could not show any difference in growth properties between normal and Huntington's chorea fibroblasts when 20% dFCS with MEM was used. This apparent difference in results could very well be explained by the use of different sera and media. The profound affect nutrition has on human fibroblasts is shown in this paper.

Huntington's chorea fibroblasts expressed a metabolic block when the culture

Figure 4.

Replating of Huntington's chorea and normal fibroblasts.

Logarithmically growing cells were plated at a density of 50,000 per 25 ml flask in 5 ml MEM with 20% dFCS. Fresh medium was added three days later. On the seventh day and at three day intervals the flasks received the indicated components in 0.5 ml volume. Control flasks had fresh media added every three days.

Seventeen days after the start of the experiment one flask was used for photography. The rest were trypsinized and counted. Fifty thousand cells were replated to test the viability and replicative capacity of cells under each experimental condition in MEM and 20% dFCS, and fed with fresh medium every three days. Two weeks later these flasks were photographed, then trypsinized and counted.

a, c, e represent normal cells that had been supplemented with (a) glycine + asparagine + glutamine; (c) with glutamine; and (e) with glucosamine respectively. Panels b, d, f represent Huntington's chorea cells that were similarly supplemented as in a, c and e. Magnification of original photomicrograph x 450.

medium is depleted and glutamine is the only source of fresh supplementation. This block can be overcome when glucosamine is supplemented to depleted media, indicating that mutant cells cannot convert sufficient glutamine to glucosamine. Normal cells that have been maintained in depleted medium but not Huntington's chorea fibroblasts do well by supplementation with glutamine, asparagine + glutamine + glycine or glucosamine.

It appears likely that the site of the metabolic block is localized to the following enzymatic step.

$$\text{L-glutamine} + \text{D-Fructose-6-P} \xrightarrow[\text{transamidase}]{\text{L-Gln D-F-6-P}} \text{glucosamine} + \text{glutamate}$$

This enzyme is the committed step in the synthesis of hexosamine and sialic acid components of membrane glycoprotein and glycolipid. (4) While the abnormal gene product in Huntington's chorea may be located in the cytoplasm the phenotype may clinically express in the neuronal membrane. This postulate would predict that the mutation of Huntington's chorea would first herald its presence in the central nervous system by an initial derangement of membrane specific functions. Membrane potential, cation and anion transport neurotransmitter synthesis, release and storage, amino acid, carbohydrate transport defects are in turn followed by destruction of neural networks.

The use of skin fibroblasts in culture to study the nutritional requirements of this specific human mutation has promise for the following possibilities. First, it allows for the meaningful and systematic search for the site of the mutated enzyme. We are obtaining additional skin fibroblasts from other cases of Huntington's chorea and controls to determine if other cell lines from affected individuals also express the reported findings of this paper. Second, the survival and reproductive capacity of the cell is challenged by selective depletion of known components from the culture medium and by corollary this process is reversed by selective supplementation of known components distal to the block. As a logical consequence the system will afford immediate opportunities to explore possible strategies of therapy of the human disease by substitution of the missing metabolite

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