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Differential labelling of UDP-*N*-acetylglucosamine in Huntington's-chorea fibroblasts

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The hypothesis that there is impaired endogenous synthesis of glucosamine 6-phosphate in Huntington's-chorea fibroblasts was tested by double labelling matched pairs of fibroblasts in culture with carrier-free $H_3{}^{32}PO_4$ and [U-^{14}C]glucosamine. The [^{32}P]-UDP-*N*-acetyl[^{14}C]glucosamine and [^{14}C]glucosamine 6-[^{32}P]phosphate of the cellular soluble fraction was isolated by charcoal column and paper chromatography. There is no quantitative difference in ^{32}P but a significant difference in ^{14}C in these two sugars in a ratio of approx. 1.5 for Huntington's-chorea fibroblasts compared with normal fibroblasts.

Huntington's chorea is an autosomal dominant human mutation affecting the central nervous system. Peripheral tissues outside the brain have been used to characterize a number of interesting differences from normal. Thus secondary skin fibroblasts in culture exhibit greater than normal protein and lipid glycosylation when labelled with [U-^{14}C]glucosamine without an apparent accompanying difference in cellular uptake of [^{14}C]glucosamine, general cellular synthesis of protein or degradation of ^{14}C-labelled macromolecules (Tourian & Hung, 1979; Hung *et al.*, 1980). One hypothesis that could explain these observed differences of protein and lipid glycosylation in Huntington's-chorea fibroblasts, compared with normal, is a differential utilization of exogenously supplied [^{14}C]glucosamine due to impaired endogenous synthesis of glucosamine 6-phosphate. This hypothesis was tested by labelling the fibroblast cellular UDP-*N*-acetylglucosamine for 72h with [^{32}P]P_i. Simultaneously, the glucosamine 6-phosphate, UDP-*N*-acetylglucosamine and *N*-acetylglucosamine were labelled with [^{14}C]glucosamine for 48h.

The [^{32}P]UDP-*N*-acetyl[^{14}C]glucosamine of the cellular soluble fraction was isolated by sequential charcoal and paper chromatography. There was no quantitative difference in ^{32}P, but a significant difference in ^{14}C content, in UDP-*N*-acetylglucosamine between Huntington's-chorea and normal fibroblasts. The ratio of ^{14}C in UDP-*N*-acetylglucosamine between Huntington's-chorea and normal fibroblasts was approx. 1.5, suggesting that endogenous synthesis of UDP-*N*-acetylglucosamine is impaired in Huntington's chorea.

Materials and methods

Materials

Skin fibroblasts GM-967, GM-1061, GM-124, GM-305 and GM-964 were obtained from the Human Genetics Mutant Cell Repository, Institute for Medical Research, Camden, NJ, U.S.A. Cell line 7904 was from a 31 year-old male Huntington's-chorea patient from our clinic. All the cultures were found to be free from pleuropneumonia-like organisms by periodic mycoplasma tests (Russell *et al.*, 1975). Eagle's minimal-essential medium and dialysed foetal calf serum were purchased from GIBCO. Nuchar-C-190 Granular, a highly activated vegetable carbon, was obtained from Division West Virginia Pulp and Paper Co., Covington, VA, U.S.A. [U-^{14}C]Glucosamine (sp. radioactivity 300Ci/mol), [^{32}P]P_i (carrier-free) and Omnifluor were purchased from New England Nuclear. Unlabelled D-glucosamine hydrochloride, UDP-*N*-acetylglucosamine, glucosamine 6-phosphate and *N*-acetylglucosamine were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A.

Growth of fibroblasts

Fibroblasts were grown in Eagle's minimal essential medium in 20% dialysed foetal calf serum as previously described (Tourian & Hung, 1977*a*,*b*). Sex, age, passage number and growth rate of Huntington's-chorea fibroblasts were matched with

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normal controls. The cell number and protein concentration of matched pairs were not statistically different at the time of harvest. The passage number of the fibroblasts includes those between the tenth and fifteenth passage.

Double labelling of cell cultures with [U-^{14}C]-glucosamine and [^{32}P]P_i

Exponentially growing cells were plated in duplicate at 3×10^5 cells/25 cm^2 flask in 5 ml of minimum essential medium and 20% dialysed foetal calf serum. Fresh medium was added on day 3. The following day 5 ml of minimum essential medium containing [^{32}P]P_i (carrier-free, at 0.5 μCi/ml) was added. On day 5, the medium was changed with 2 ml of ^{32}P-containing medium also containing [U-^{14}C]-glucosamine at 2.5 μCi/ml and incubated for 48 h.

Harvesting cell monolayers

The culture medium was discarded and the monolayers were washed twice with 10 ml of cold phosphate-buffered saline. The cells were scraped with a rubber 'policeman' in 0.5 ml of cold phosphate-buffered saline containing 1 mM-phenylmethanesulphonyl fluoride. Portions were removed for total cellular incorporation of [U-^{14}C]glucosamine and protein determination. An equal volume of cold 10% trichloroacetic acid was added to the remaining cell suspension, mixed and left in an ice bath for at least 15 min. The suspension was then centrifuged at 1500 *g* for 10 min. The supernatant was collected and the precipitate was washed with the same volume of cold 5% trichloroacetic acid and then centrifuged once more. Both supernatants were combined as the trichloroacetic acid-soluble fraction and the precipitate was dissolved in 0.5 ml of 0.1 M-NaOH as the trichloroacetic acid-insoluble fraction. Portions were removed for evaluation of radioactivity in an Intertechnique SL-4000 liquid-scintillation counter, using 0.4% Omnifluor in a mixture of toluene/Triton X-100 (2:1, v/v).

Charcoal column chromatography

The trichloroacetic acid-soluble material was added on to a charcoal column (0.8 cm × 5 cm) (Bartlett, 1959). The column was sequentially eluted with water, 20% ethanol and 50% ethanol in 2 M-NH_3 in that order. Four 2 ml fractions were collected from each elution. All the fractions in each elution were pooled, freeze-dried and re-dissolved in a small known volume of water for further analysis.

Paper chromatography of sugar phosphates and nucleotide sugars

Nucleotides, nucleosides and glucosamine and its derivatives in the fractions of trichloroacetic acid-soluble material from charcoal chromatography were further separated by ascending chromatography on Whatman 3MM paper for 18 h in the following solvent systems: solvent 28, 1 M ammonium acetate (pH 5.0)/95% ethanol (3:7, v/v); solvent 9, saturated $(NH_4)_2SO_4$/0.05 M-phosphate buffer (pH 6.0)/isopropyl alcohol (79:19:2, by vol.) (Plagemann & Erbe, 1973). Authentic standards of ATP, UDP-*N*-acetylglucosamine, *N*-acetylglucosamine 6-phosphate, *N*-acetylglucosamine, glucosamine 6-phosphate and glucosamine were used for identification. Developed chromatograms were cut into $\frac{1}{2}$ inch strips and eluted with 0.5 ml of water and the eluates, along with the paper, were counted in a scintillation counter for radioactivity. The radioactivity in ^{32}P and ^{14}C was evaluated by adjusting the window openings to count each isotope independently of the other.

Results and discussion

Incorporation of [^{14}C]glucosamine into hexosamine and nucleotide sugars in the fibroblasts

At the end of 48 h of incubation with [^{14}C]-glucosamine and 72 h with [^{32}P]P_i, 55–66% of the total cellular ^{14}C-containing material was in the acid-soluble fraction of cells. The double-labelled ^{32}P- and ^{14}C-containing acid-soluble material from matched pairs of fibroblasts was put on charcoal columns and sequentially eluted with water, 20% ethanol and 50% ammoniacal ethanol. Neutral sugars, sugar phosphates, nucleotides and nucleotide sugars were sequentially eluted, followed by paper chromatography of each fraction. There was complete recovery of the radioactivity of all of the acid-soluble material from phenotypes of fibroblasts.

The total amount of ^{32}P in UDP-*N*-acetylglucosamine was identical for both Huntington's-chorea and normal fibroblasts, whereas the total ^{32}P in UDP-*N*-acetylglucosamine did not vary from experiment to experiment or between normal and Huntington's-chorea fibroblasts. The total ^{14}C in UDP-*N*-acetylglucosamine varied somewhat from experiment to experiment but the ratio between Huntington's-chorea and normal fibroblasts was always approx. 1.5 (Table 1). Therefore we looked in a similar fashion at the ^{32}P- and ^{14}C-labelling pattern of [^{14}C]glucosamine 6-[^{32}P]phosphate in two matched pairs of Huntington's-chorea and normal fibroblasts. The ^{32}P in [^{14}C]glucosamine 6-[^{32}P]-phosphate was identical in amount in Huntington's-chorea and normal fibroblasts, whereas ^{14}C in [^{14}C]glucosamine 6-[^{32}P]phosphate was always higher in Huntington's-chorea fibroblasts in two matched pairs of Huntington's-chorea and normal fibroblasts with a ratio of approximately 1.5 (Table 2 and Fig. 1).

The culture medium for the growth of human fibroblasts is a complex nutritional preparation due

Table 1. *The ratio of ^{32}P and ^{14}C in [^{32}P]UDP-N-acetyl-[^{14}C]glucosamine in Huntington's-chorea (HC) and normal (NL) fibroblasts*

Portions from fractions collected through charcoal column chromatography in eluates of water were applied to Whatman 3MM paper and were separated in solvent 9. The developed chromatograms were cut into ½ inch strips and radioactivity was counted in dual-isotope windows for both ^{14}C and ^{32}P, which were adjusted with unquenched standards to minimize spillover between isotopes and actual radioactivity and each isotope in each sample vial was corrected to account for the efficiency and percentage of cross-contribution from each isotope. For both ^{32}P and ^{14}C, the radioactivity in Huntington's-chorea fibroblasts compared with that in normal fibroblasts is shown as a ratio in parentheses.

Cell lines	$10^{-5} \times {}^{32}P$ radioactivity (c.p.m./mg of protein)		$10^{-4} \times {}^{14}C$ radioactivity (c.p.m./mg of protein)	
HC 1061	4.85	(0.97)	39.93	(1.58)
NL 967	4.99		25.26	
HC 305	5.15	(1.04)	29.30	(1.71)
NL 124	4.95		17.18	
HC 305	5.54	(0.90)	18.79	(1.62)
NL 124	6.13		12.78	
HC 7904	4.97	(1.11)	14.87	(1.45)
NL 964	4.48		9.21	

Table 2. *The ratio of ^{14}C and ^{32}P in [^{14}C]glucosamine 6-[^{32}P]phosphate and ^{14}C in N-acetyl[^{14}C]glucosamine in Huntington's-chorea (HC) compared with normal (NL) fibroblasts*

Portions from fractions collected through charcoal column chromatography in eluates of 50% ethanol in 2 M-NH_3 elution were applied to Whatman 3MM paper and were separated in solvent 28. The developed chromatograms were processed as described in the legend to Table 1. The radioactivities in Huntington's-chorea fibroblasts compared with normal fibroblasts are given as ratios in parentheses.

	$10^{-5} \times$ Radioactivity (c.p.m./mg of protein)					
	[^{14}C]Glucosamine 6-[^{32}P]phosphate				^{14}C in *N*-acetyl[^{14}C]-glucosamine	
	^{32}P		^{14}C			
HC 305	19.72	(0.92)	6.08	(1.73)	3.29	(1.30)
NL 124	21.51		3.52		2.53	
HC 7904	13.20	(1.11)	3.40	(1.42)	1.59	(1.21)
NL 964	11.90		2.40		1.31	
HC 305	15.10	(1.09)	4.74	(1.60)	2.44	(1.27)
NL 124	13.83		2.96		1.92	

to the presence of serum. Foetal calf serum has at least five known glycosidase activities that can hydrolyse the sugars from serum glycoproteins.

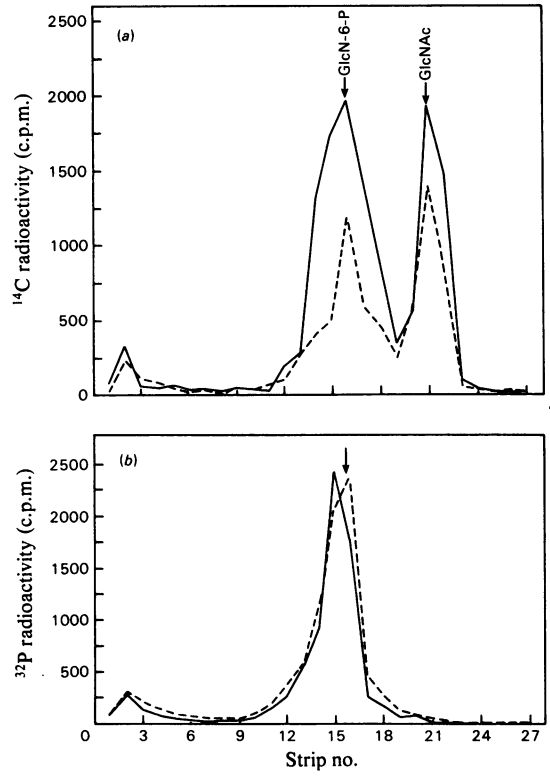

Fig. 1. *The quantitative profile of [^{14}C]glucosamine 6-[^{32}P]phosphate and N-acetyl[^{14}C]glucosamine from the water eluate of the charcoal column chromatogram of Huntington's-chorea and normal fibroblasts*

Pooled fractions of the water eluate from charcoal column chromatography were separated by paper chromatography in solvent 9 as described in the Materials and methods section. Each strip cut from the paper chromatogram was counted by liquid-scintillation spectrometry for both ^{14}C and ^{32}P radioactivity. The window openings for each isotope were adjusted to count each isotope exclusively. The profile of ^{14}C radioactivity in the paper chromatogram and of ^{32}P radioactivity in the same paper chromatogram are shown. Abbreviation used: GlcN-6-P, glucosamine 6-phosphate. ——, Huntington's-chorea fibroblasts; – – – –, normal fibroblasts.

These sugars will then be taken into fibroblasts (Bloch *et al.*, 1977). Since human fibroblasts cannot grow without serum, the direct determination of hexosamines and UDP-*N*-acetylglucosamine under serum growth conditions will not reflect the true endogenous rates of synthesis of these sugars of interest. The removal of small molecules and glycosidase activities from foetal calf serum can be achieved by dialysis, heating the serum for 30 min at 65°C followed by Sephadex G-50 gel filtration.

Supplementation of the culture media with such serum in addition to serine results in an equivalent growth condition to that of non-treated serum. However, for indeterminate reasons the glycosylation of proteins in fibroblasts is markedly decreased to less than 10% of control serum indicating that the Sephadex G-50 step removes from the serum the components that are essential for optimum glycosylation of fibroblasts (Tourian & Hung, 1978). Additionally the glycosylation of macromolecules varies with the cell cycle (Tenner *et al.*, 1977). The method of double labelling hexosamines and UDP-*N*-acetylglucosamine in the acid-soluble compartment of the fibroblasts under equilibrium conditions permits an estimate of the endogenous and exogenous utilization of glucosamine and UDP-*N*-acetylglucosamine by the fibroblasts of differing genotypes.

In the case of utilization of glucosamine, it is the relative contribution of fructose 6-phosphate and the exogenous radioactive glucosamine to the glucosamine 6-phosphate pool that determine the ultimate specific radioactivity of UDP-*N*-acetylhexosamine formed from labelled glucosamine. These relative contributions are in part regulated by the relative concentration of glucose and glucosamine (in the medium), so that the contribution of fructose 6-phosphate to glucosamine 6-phosphate decreases as the amount of glucose in the medium decreases to less than 1 mM even though the UDP-*N*-acetylglucosamine pool remains constant as might be expected (Kim & Conrad, 1976).

Since Huntington's chorea is an autosomal dominant mutation, the gene-dosage principle would predict that the locus for the hemizygous Huntington's-chorea gene would express 50% of a given enzyme or structural protein in a normal fashion, since one of the alleles for the mutated site is normal. Thus any observed difference from normal cannot be an absolute but a relative one. The method of double labelling hexosamines of fibroblasts with [^{32}P]P_i and [^{14}C]glucosamine distinguishes the exogenous labelled sugars from the endogenous metabolic flow of hexosamines in the soluble compartment of the cells and suggests that glucosamine 6-phosphate synthesis is impaired in Huntington's-chorea fibroblasts. Whether these observations of abnormal hexosamine metabolism in Huntington's-chorea fibroblasts reflect a primary phenotypic alteration or are secondary to a more fundamental primary cellular process awaits the direct measurement of glucosamine 6-phosphate synthetase in secondary fibroblasts in culture.

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