Membrane fluidity measurements in peripheral cells from Huntington's disease patients

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SUMMARY The primary gene defect of Huntington's disease is believed to involve the membrane of some peripheral cells. The membrane fluidity of skin fibroblasts, erythrocytes and leucocytes was measured by fluorescence polarisation of a lipid specific probe, 1,6 diphenyl, 1,3,5 hexatriene. No significant difference between controls and Huntington's disease patients could be demonstrated.

Despite considerable research, the primary gene product of Huntington's disease (HD) has yet to be defined. The greatest problem is the inability to diagnose potential carriers of the disease, which, coupled with the fact that 50% of the offspring of an affected individual will contract the illness, makes it imperative that a reliable diagnostic technique be developed. The strategy of investigation into the disease has, however, changed dramatically in the last few years. Early studies involved primarily the pathology and biochemistry of the brain after death; however, since that time, the search has been extended to other peripheral tissues, based upon the concepts that in autosomal dominant diseases, the abnormal gene product is believed to be present (though not always completely expressed) in all cells of the body and secondly, that some neuropsychiatric disorders may be associated with membrane defects.

Menkes and Stein¹ were among the first to claim differences in growth characteristics of Huntington fibroblasts. A review by Appel² described other studies which suggested that fibroblasts grew to greater density than normal and in addition appeared to be unusually sensitive to the lipid content of the medium. Tourian *et al.*,^{3,4} have suggested that HD fibroblasts are dependent upon glucosamine for cell survival in culture, display delayed adhesion to a plastic substrate and are also sensitive to excess glutamine. The increased growth rate and reduced contact inhibition of

cultured HD fibroblasts is also a feature of transformed cells, which is of interest, since cellular transformation has also been observed in the fibroblastic-type cells from brain tissue of individuals with certain degenerative diseases of the CNS, for example Kreutzfeldt-Jakob disease.⁵ If the transformation idea is true, however, it would be reasonable to suppose that changes would also be expressed at the cell membrane and indeed such changes have been claimed to occur in HD cells.

Electron spin resonance investigations by Butterfield and Markesbury,⁶ using a sulphhydryl group-specific probe MAL-6, have indicated an alteration in the protein organisational structure of HD erythrocyte membranes. In addition, an elevation in the membrane-bound Na⁺K⁺ ATPase enzyme activity, was also reported.⁷ However Pettegrew *et al.*,⁸ were unable to demonstrate any significant difference in levels of high energy phosphates in HD or controls, with a nuclear magnetic resonance (NMR) study, thus the significance of elevated Na⁺K⁺ ATPase, remains to be determined. Pettegrew *et al.*^{9,10} reported that the cell membranes of both skin fibroblasts and erythrocytes from HD patients have different fluorescent characteristics from normals. They used the fluorescent probe 1,8-anilino naphthalene sulphonic acid (ANS) which when bound to specific membrane regions, gave a 30-fold increase in intensity of excitation and emission spectra in HD cells, a shift in the emission maximum and an increase in polarisation of the emission spectrum. Continued subculture of HD fibroblasts to higher passages showed a change in these findings towards normal.

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In this paper we have examined the fluorescent polarisation characteristics of the cell membranes of skin fibroblasts, erythrocyte ghosts, and lymphocytes, from both HD and controls. We used the fluorescent probe 1,6 diphenyl, 1,3,5, hexatriene (DPH), in an attempt to establish whether there might be a difference in membrane fluidity between the cells of the two types of individual. DPH is one of the most frequently employed probes for measurements of fluorescence polarisation. A monochromatic, vertically polarised light source provides maximum excitation and the emission intensity is detected through an analyser orientated both parallel and perpendicular to the polarisation direction of the exciting light source. The degree of fluorescence polarisation can be represented by the formula:

$$P = \frac{I_{ii} - I_i}{I_{ii} + I_i}$$

Where I_{ii} = Intensity of the fluorescent light, polarised parallel to the exciting beam.

Where I_i = Intensity of the fluorescent light, polarised perpendicular to the exciting beam.

The polarisation value P is directly proportional to the microviscosity of the cell membranes, so that as P increases, the membranes become more rigid and less fluid. DPH itself is a molecule with an ellipsoidal form that resembles a rotating rod, with the advantage that the absorption and emission oscillators lie along the long axis of the fluorophore, hence only those rotations at 90° to this axis will be effective in depolarising the fluorescence emission. DPH has an absorption maximum of 355 nm and in aqueous solutions, gives almost no fluorescence, but when in a lipid system, gives approximately a 1000-fold increase in fluorescence. It is believed to be attached to the hydrocarbon region of the lipid layer in the cell membrane and to be almost evenly distributed. The probe has been widely used in membrane research to measure the membrane fluidity of normal and leukaemic lymphocytes of both humans and animals,¹¹⁻¹⁵ normal and transformed malignant fibroblasts,¹⁶⁻¹⁹ to demonstrate membrane interactions between contacting cells,²⁰ to study variations in membrane fluidity during the cell cycle,²¹⁻²² and as an assay of phospholipid vesicles.²³

Materials and methods

Cells

Fibroblasts: skin biopsies from eight adult and three juvenile HD patients and 11 normal controls were dissected into small pieces and put under sterile

coverslips in Corning 38 mm Petri dishes. Hams F10 medium (Flow Labs) supplemented with 20% foetal bovine serum (Flow Labs), 20 mM glutamine, hypoxanthine 136.1 mg/100 ml PBS, penicillin 500 000 IU/l and streptomycin 500 000 mcg/l was used as culture medium. The cells were incubated for 2 weeks at 37°C until a suitable outgrowth had occurred. The coverslips were then removed and placed with the cell layer uppermost in new Petri dishes and fresh medium added to all dishes. The medium was refreshed three times per week. When confluency was reached, after about 5-6 weeks, the fibroblasts were subcultured and plated out into 25 cm² Falcon flasks (passage 1). The flasks were then incubated until confluent, again subcultured (passage 2) and plated out with a 1:2 split into 75 cm² Falcon flasks and left until confluent (passage 3). Cells were grown for a further week, subcultured into glass Roux bottles and grown to confluence (passage 4). When confluent the cells were trypsinised, subdivided into 1 ml glass ampoules with cryopreservative and stored in liquid N₂ at passages varying from 4-7.

Staining details

Fluorescence labelling of cells: the day before the experiment, three confluent flasks of fibroblasts per individual were washed once in warmed Earles medium and trypsinised with $\times 2$ EDTA trypsin for 10 minutes until all the cells were detached. The cells were then thoroughly resuspended with a Pasteur pipette until completely homogenous and the action of the enzyme stopped by the addition of 5 ml fresh complete medium. The fibroblasts were centrifuged at 1200 rpm for 10 minutes, the supernatant removed to 1 ml and the cells resuspended in fresh Hams medium without serum, and put into siliconised glass tubes. The tubes were slowly rotated overnight at 37°C to reduce the possibility of fibroblast attachment to the walls of the test tubes, to maintain them in a rounded form and to allow the cells sufficient time to recover from any deleterious effects of the trypsin.

A stock solution of 2 mM DPH (1,6,diphenyl, 1,3,5, hexatriene) Koch Light Laboratories, Colnbrook, England) was prepared in tetrahydrofuran. A working solution, was freshly prepared before each measuring session, that is 100 μl DPH solution in tetrahydrofuran was added rapidly to vigorously stirred 100 ml phosphate buffered saline and kept under bubbling nitrogen for 30 minutes to ensure complete evaporation of the tetrahydrofuran. The final solution was faintly opalescent and was checked for its fluorescence in the microviscometer before use. The fibroblasts were then washed twice in warmed PBS, stained with the DPH for 30 minutes at 37°C , put into quartz measuring cuvettes and the polarisation readings in triplicate were taken immediately.

Erythrocytes: peripheral blood was obtained by venepuncture from seven patients and six normal controls with EDTA as anticoagulant. "Ghost" cells were prepared by the method of Dodge *et al*²⁴ and kept overnight in PBS at 4°C . The next day, the cells were

washed once in warm PBS, stained for 30 minutes at 37°C, washed twice more and measured.

Leucocytes: 10 ml of peripheral blood was obtained in heparin from two patients and two controls, mixed with Hanks balanced salt solution and 14 ml Ficoll-Hypaque solution and allowed to stand. When settled out, the suspension was centrifuged for 15 minutes at 1800 rpm and the supernatant discarded. The buffy coat was removed and washed thoroughly with Hanks to ensure complete removal of the Ficoll. Cell samples were taken, stained with Turks Fluid and the cells counted in a Bürcher haemocytometer. The cells were stored overnight in RPMI 1640 medium and the next day washed once in PBS, stained for 30 minutes with DH, washed twice more and measured.

Fluorescence measurements

Fluorescence polarisation and intensity were measured in an instrument especially adapted from a Zeiss PMQ-2 spectrophotometer. Light from a 100W mercury vapour lamp of excitation 365 nm, was passed through a rotatable sheet polariser. The fluorescence intensity was measured simultaneously both parallel and perpendicular to the exciting beam by photomultiplier tubes, after passing through an aqueous 2M NaNO₂ solution as cutoff filter for all wavelengths of less than 390 nm. The temperature was controlled by thermoelectric elements (Cambion Model 3959-01, Cambridge Thermionic Corp, Cambridge, Mass) and held to $\pm 0.1^\circ\text{C}$ over the range 0°C – 40°C . Polarisation values were calculated by the formula previously mentioned.

Results

The mean polarisation values obtained for Huntington fibroblasts (table 1), varied between 0.24–0.29 and for control cells between 0.25–0.28, with an overlap of values among all groups. No signifi-

cant difference in mean values between any matched pair of patient/control samples was detectable, although the HD fibroblasts showed a greater range of values in polarisation values, ie 0.2227–0.3023, compared with 0.2447–0.2831 for controls. The polarisation values for cultures of high passage number, did not differ significantly from those at lower passage for both patient and control groups. In so far as the age of biopsy donors was concerned, there appeared to be little difference in polarisation values between the five juvenile cases and the adult cases.

The adult donors were all within a restricted age range of 30–50 years; older individuals were not used because of the possible variation in growth characteristics of senescent skin fibroblasts. Fibroblast cell lines from two patients and two controls were also obtained from the Cell Repository, Camden, New Jersey, matched for sex and age, to act as reference samples. Again, no appreciable difference was observed between cultures established from our biopsies and those obtained from Camden. Inspection of standard deviation (SD) values (tables 1 and 2), suggests a greater heterogeneity in the HD population than in normals. This was confirmed by application of the variance ratio test which showed that for both fibroblasts and erythrocyte ghost cells, the calculated F value approximated to a 5% significance difference.

Fibroblast suspensions in triplicate were prepared from confluent Falcon flasks as described in *Materials and methods* and the polarisation values observed and measured on three different occasions over a 2-week period (table 3). It would appear that the polarisation values vary by a fac-

Table 1 Polarisation (P) values of human fibroblasts in suspension with the fluorescent probe 1,6Diphenyl, 1,3,5,Hexatriene (DPH)

Huntington patients						Controls					
Name	Age (yr)	Sex	Passage number	(P)	SD	Name	Age (yr)	Sex	Passage number	(P)	SD
JHN	30	M	10	0.2467	0.0055	FN	28	F	10	0.2533	0.0109
HDH	43	F	7	0.2586	0.0047	AD	43	M	7	0.2718	0.0035
JVH	40	M	7	0.2544	0.0176	VAH	39	F	7	0.2571	0.0178
MH	11	F	4	0.2907	0.0044	SB	13	F	4	0.2800	0.0054
MIM	47	M	4	0.2701	0.0065	G	42	M	4	0.2591	0.0087
SEB	36	F	4	0.2456	0.0060	MJB	36	F	4	0.2600	0.0042
RH	6	M	4	0.2409	0.0040	LT	9	F	4	0.2635	0.0072
JWH	32	M	3	0.2727	0.0039	GJB	28	F	13	0.2558	0.0041
MM	13	M	10	0.2459	0.0056	SB	13	F	4	0.2800	0.0054
GM-1170	35	F	9	0.2913	0.0084	GM-2153	40	F	9	0.2693	0.0046
GM-1085	44	M	9	0.2709	0.0029	GM-2169	52	M	9	0.2697	0.0089

Total no patients=11.
Mean of all P values ($\bar{x}$)=0.2625.
Standard error of mean=0.0179.
Variance ratio: P=0.05 where F=3.

Total no controls=10.
Mean of all P values ($\bar{x}$)=0.2639.
Standard error of mean=0.0085.

Table 2 Polarisation values of erythrocyte ghosts with the fluorescent probe 1,6-Diphenyl, 1,3,5,Hexatriene

Huntington patients					Controls				
Name	Age (yr)	Sex	P value	SD	Name	Age (yr)	Sex	P value	SD
SEB	29	F	0.3175	0.0084	MJB	37	M	0.3222	0.0060
LB	28	M	0.3340	0.0138	—	—	—	—	—
HDH	44	F	0.3126	0.0124	AD	44	M	0.3142	0.0047
EJS	29	F	0.3310	0.0015	WE	28	M	0.3173	0.0014
JHGK	34	F	0.3024	0.0015	GG	42	M	0.3102	0.0014
WPvL	65	F	0.3216	0.0043	LW	52	M	0.3257	0.0060
DK	58	M	0.3200	0.0048	LW	52	M	0.3126	0.0045

Total no patients=7.
Mean of all P values ($\bar{x}$)=0.3199.
Standard error of mean=0.0107.
Variance ratio: P=0.05 where F=3.

Total no controls=6.
Mean of all P values ($\bar{x}$)=0.3170.
Standard error of mean=0.0059.

Table 3 Repeated polarisation measurements of fibroblasts with DPH

Name	Age (yr)	Sex	Original measurement	1st repeat	2nd repeat	3rd repeat
MH	12	F	0.29±0.004	0.25±0.005	0.23±0.0045	0.23±0.005
RH	7	M	0.24±0.004	0.246±0.005	0.21	0.23±0.01
MM	15	M	0.24±0.005	0.27	0.214±0.005	0.236±0.005
1170	34	F	0.29±0.008	0.242±0.0084	0.24	0.254±0.005

tor of +0.05, when measured at different times. This needs to be taken into account when assessing the significance of differences between cell types. Cell viability tests, using trypan blue, showed the fibroblasts to have a viability of 90–95% after the overnight rotation in the siliconised tubes.

Table 2 shows the polarisation values obtained for the erythrocyte ghosts, which were higher than those from fibroblasts and within closer limits, probably due to the greater homogeneity of the red cells. However, no significant difference was observed between Huntington cell and normals, nor between age or sex of the individuals. Polarisation values for leucocytes showed no discernible difference between Huntington patients and controls (see table 4).

Table 4 Polarisation values of leucocytes with the fluorescent probe 1,6-Diphenyl, 1,3,5,Hexatriene

Name	Age (yr)	Sex	Type	P value	Cell counts $\times 10^6/\text{ml}$
LW	52	M	Control	0.28	2.2
MK	55	F	Control	0.28	2.5
DK	58	M	HD	0.27	1.96
WPL	65	F	HD	0.28	2.3

Discussion

There is evidence to support the idea of a general membrane defect in Huntington's disease.^{2,4 6-10} The fluorescent probe DPH has been widely used in the study of the dynamics of the hydrocarbon

region of the surface membrane lipid bilayer.^{11 25-28} We have used DPH to investigate the membrane fluidity of the cell membranes of fibroblasts, leucocytes and "ghost" erythrocytes from both Huntington patients and normal controls. No significant difference was found between patient and normal controls in any of the different cell types used, a finding confirmed by Kraus and Williams for lymphocytes (unpublished) and by Abood and Butler²⁹ for erythrocyte ghost cells. The magnitude of our measurements corresponds with the findings obtained by other investigators on similar cell types.

The majority of fibroblast cultures used were in early passage (3 to 4) at the time of measurement and the remainder, at late passage (7 to 13) did not appear to differ significantly in their polarisation values. Hence passage number would not seem to be a critical factor, also confirmed by Haggerty *et al.*³⁰ In contrast, Pettegrew *et al.*^{9 10} found that the corrected spectra of Huntington fibroblasts after staining with the fluorescent probe 1,8-anilosulphonic acid, were indeed grossly different in excitation and emission intensity at early passage but this difference was found to have disappeared by passage 10.

Independently established fibroblast cultures from the Camden Cell Repository were found to have similar polarisation values and growth characteristics to the cultures established in our laboratory.

The results shown in this study are expressed in

terms of polarisation values only, which are in turn, directly proportional to viscosity, that is the higher the P value, the higher viscosity, hence the lower the fluidity of the membrane and vice versa. No depolarisation corrections were made to the polarisation values we obtained because the intensity of the fluorescent signal was high enough to make the effects of light scattering or background values, negligible.²¹ We have measured fluorescent anisotropy of skin fibroblasts in suspension, subsequent to trypsinisation, in preference to measurement of confluent cells grown on suitably sized coverslips, as advocated by other authors.^{9 10 21} Our pilot studies showed that after 3–7 days in culture, both confluent and non-confluent fibroblasts on glass, produced extremely low fluorescence polarisation values in comparison to suspension cultures. The glass coverslips, when inserted into the quartz cuvettes of the spectrophotometer, were found to be covered with a milky white deposit which was sufficiently turbid to prevent most of the fluorescent signal reaching the photomultiplier tubes. The explanation of this observation is not known.

The trypsinisation of confluent fibroblasts was found to provide a more homogenous suspension with less debris and a greater cell yield, than that obtained by scraping the cells with a rubber policeman. However, de Laat *et al.*²¹ suggested that trypsin treatment increased the microviscosity of the cell membrane whereas Shinitzky and Inbar,¹¹ Inbar *et al.*¹⁶ and Levanon *et al.*³¹ could find no such difference in polarisation after the action of this enzyme.

In conclusion, fluorescent polarisation measurements of the plasma membranes of skin fibroblasts, red cells and leucocytes showed no significant difference between Huntington patients and control samples. They are therefore concordant with the opinion of Butterfield *et al.*^{6 7} and Abood and Butler,²⁹ that alteration in the lipid fraction of the membrane is not the genetic defect involved in the disease. It is possible that the greater heterogeneity demonstrated by HD patients may be caused by extraneous effects such as drug therapy, weight loss etc. The uncertainty surrounding the exact location of the fluorescent probe in the cell membrane, does not necessarily invalidate our results since Andersen,²⁵ has suggested that an alternative way of using fluorescent probes is to regard the measured properties merely as diagnostics for observing membrane transitions, even though details of the actual probe location within the membrane are not known. Further work is in progress to examine the protein configuration of

membranes from erythrocyte ghosts in Huntington's disease, using the electron spin resonance technique described by Butterfield and Markesbury.⁶

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