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Skin Fibroblasts in Huntington Disease

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SUMMARY

We previously reported that skin fibroblasts with Huntington disease (HD) grew to higher maximal densities and, at early culture passages, attained more population doublings per week than did fibroblasts from control individuals. We also noted that HD cells were smaller and that larger colonies developed from single cells. In view of discrepant results reported from replications of the above studies, we undertook extensive blind studies with 10 coded pairs of HD and control cells in which all the skin biopsies were obtained by the same method, and the HD and control cells were grown identically at all times. No significant differences were found between HD and control cells in any of the above parameters in the current study. Some of the possible reasons for our failure to reproduce the previous results are discussed, chief among them may be the different treatments to which the HD and control cells might have been subjected prior to coming to our hands and the utilization of skin samples from different regions of the body.

INTRODUCTION

Huntington disease (HD) is a hereditary neurological disorder of which the basic genetic defect and pathogenesis are unknown. Since biopsy material from brain is not readily available for study, fibroblasts grown in vitro from skin biopsies are being investigated with the hope that the HD gene might be expressed in these cells. It was reported that skin fibroblasts from HD patients grow poorer than those from controls [1, 2]. On the other hand, we found that skin fibroblasts from HD patients

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grew to a significantly higher maximal density than did those from control subjects of approximately the same age [3]. This observation subsequently was confirmed in three other laboratories [4–6]. In addition, we observed that HD cells, at early culture passages, attained more population doublings per week than did control cells. During continued growth in vitro, population doublings per week decreased for both HD and control cells but decreased at a faster rate for HD cells [7]. We also noted that HD cells were smaller and that larger colonies developed from single cells (our unpublished observations, 1979). Reports by other investigators who did not find significant differences in the growth characteristics of HD and control fibroblasts [8] prompted us to undertake extensive blind studies with 10 coded pairs of HD and control cells in which all skin biopsies were obtained by the same method and the fibroblasts were grown identically. The parameters examined (maximal cell density, population doublings per week, cell volume, and colony size) were those that in previous studies in our laboratory apparently had differentiated HD from control cells. Current results showed variations among cell lines, but no significant differences between HD and control cells were found. Possible reasons for the discrepant findings will be discussed.

MATERIALS AND METHODS

Skin biopsies were obtained in pairs, each pair consisting of a biopsy from a patient with HD and from an age- and sex-matched healthy control. The biopsy site was on the leg over the gastrocnemius muscle. The skin was disinfected with 70% isopropyl alcohol and infiltrated with 1% lidocaine without epinephrine. When anesthesia was attained, a 2- to 3-mm-thick piece of skin was removed with a 4-mm diameter sterile punch and placed in sterile tissue culture medium. From this point on, paired HD and control biopsies and cultured cells received identical treatment. All biopsies were coded and the codes submitted to Dr. Milton Wexler, president of the Hereditary Disease Foundation. Nine of the pairs were obtained by Dr. Anne Young at the University of Michigan Medical Center, and one pair was furnished by Dr. David Comings of the City of Hope National Medical Center. All biopsies were prepared for tissue culture within 24 hrs after excision. They were cut into 20 to 24 pieces, and equal numbers were placed in each of two 60-mm diameter culture dishes with 0.8 ml nutrient medium per dish. The explants were incubated and fed as described [3]. The basal nutrient medium was Eagle's minimum essential medium (MEM) with Earle's salts and 25 mM Hepes buffer (Gibco, Grand Island, N.Y., cat. no. 380-2360). Complete medium was prepared fresh weekly by adding 4 mM of L-glutamine, 100 U/ml of penicillin G, 100 μg/ml of streptomycin sulfate, and 10% or 20% fetal calf serum. The same lot of serum was used throughout the study. After 4 weeks, fibroblasts were removed from the dishes with trypsin-EDTA [3], and 2.5×10^5 cells were plated per 60-mm diameter culture dish with 5 ml medium. Further subcultures, with 2.5×10^5 cells per dish, were made every 7 days. Three or 4 days after each subculture, the cells were fed by replacing the medium. Population doublings were calculated for each week between subcultures as reported [7]. For maximal cell density and cell volume, cultures were fed again after 7 days and counted after 10 to 12 days. At that time, cells were detached from the dish with trypsin-EDTA, and single cell suspensions were prepared in buffered isotonic saline. Total number of cells and frequency distributions of cell volume were determined with a Coulter electronic counter that had been calibrated with 20-μm diameter polystyrene beads, and average cell volumes and standard deviations were calculated.

In studies of cell colony formation, 250 single cells were plated into a 100-mm diameter dish with 14 ml medium. After 7 days, the medium was replaced, and after 14 days, cell colonies that had developed were fixed with 10% formalin and stained with 1% crystal violet in water.

Growth of epithelial cells was estimated by determining the average diameter of the epithelial sheets that surrounded the majority of the explant pieces at the first culture passage. The trypsinization procedure removed only fibroblasts. The epithelial sheets remained attached to the dish and were fixed and stained as above. Numbers of fibroblasts per explant piece were calculated by dividing the fibroblasts per dish at the first culture passage by the number of explant pieces that were attached to the dish at that time.

All cell lines were examined for mycoplasmal contamination by determining the ratio of uptake of uridine to uracil [9] and found to be free of such contamination. A culture infected with *Mycoplasma hyorhinis* served as a positive control.

RESULTS

A summary of the results is presented in table 1. At all times, the first four pairs were grown in medium containing 10% fetal calf serum, the remainder with 20% serum from the same lot. Information on the texture of the biopsies was recorded by the technician who explanted them. Tissue was described as being hard, crunchy, brittle when cut, or soft. The means and standard deviations of the measurements given were obtained from the results, examples of which are shown for three HD-control pairs (figs. 1–3). Maximal density, population doublings per week, and colony size are measures of the proliferative capacity of the fibroblasts. Fibroblasts per explant piece at passage 1 also may reflect proliferative capacity or the number of fibroblasts in the biopsy tissue capable of adapting to growth in vitro.

It appeared from our previous work that, by comparison with control fibroblasts, the HD cells attained higher maximal densities, showed more population doublings per week, possessed smaller cell volumes, and produced larger colonies from single cells. In eight of the 10 pairs studied, all four of the assumed criteria for HD were met by one member of the pair, and in two instances, three of four criteria were attained. However, only four of the 10 pairs of the samples showing enhanced proliferative capacity were found to come from patients with HD. The means and variances of the values for the HD fibroblasts and controls also were not significantly different. Maximal density, population doublings per week, and diameter of colonies were greater in both HD and control cells when grown in medium with 20% serum than with 10% serum ($P < .001$ to $P < .05$), but fibroblast volumes were not significantly different in the two serum concentrations. These results clearly show that increased growth and decreased cell volume of skin fibroblasts are not predictive for HD.

In the five pairs in which a difference was noted in the texture of the biopsy tissue, the "soft" tissue description correlated with the formation of smaller epithelial sheets, higher values for the three parameters measuring fibroblast growth, and smaller fibroblast volume. Two HD biopsies were described as being softer, while three were harder than their matched controls.

DISCUSSION

Our study suggests that previously observed differences between HD and control skin fibroblasts are not reproducible. What are the possible causes? Our work has been performed with fibroblasts: cells thought to be connective tissue elements that make collagen and other intercellular substances. Although tissue culture fibro-

TABLE 1

COMPARISON OF PROPERTIES OF HD AND CONTROL FIBROBLASTS

	BIOPSY PAIR	AGE	SEX	TEXTURE OF BIOPSY	HD CORRECTLY PREDICTED	DIAMETER OF EPITHELIUM AT PASSAGE 1 (mm)	FIBROBLASTS				
							Cells/explant piece at passage 1* ($\times 10^4$)	Maximal density† (cells/cm^2 $\times 10^4$)	Population doublings/wk†	Diameter of cell colonies‡ (mm)	Cell vol§ (μ^3)
1	HD	66	F	...	...	...	2.31	5.87 ± 0.69	1.64 ± 0.31	1.61 ± 0.67	4261
	Control	66	M	...		...	2.86	5.47 ± 0.73	1.80 ± 0.26	1.79 ± 0.71	3822
2	HD	34	F	...	...	5.39 ± 1.24	2.47	6.45 ± 0.76	2.16 ± 0.43	2.46 ± 0.85	5264
	Control	32	F	...		3.00 ± 0.87	3.00	8.03 ± 0.53	2.33 ± 0.21	2.70 ± 1.17	4732
3	HD	34	M	Soft	+	2.58 ± 0.76	3.93	7.82 ± 1.24	2.29 ± 0.32	3.85 ± 1.50	3688
	Control	39	M	Hard		5.43 ± 2.09	2.71	5.30 ± 0.60	1.69 ± 0.32	2.86 ± 1.19	4853
4	HD	46	M	Soft	+	4.46 ± 1.74	3.87	6.80 ± 1.15	1.88 ± 0.15	3.26 ± 1.38	4113
	Control	47	M	Hard		4.94 ± 2.15	2.43	5.04 ± 1.98	1.86 ± 0.36	2.41 ± 0.91	5413
5	HD	33	M	Hard	...	6.08 ± 2.36	3.25	9.36 ± 1.79	2.41 ± 0.34	2.96 ± 1.06	4437
	Control	31	M	Soft		2.63 ± 0.95	3.89	11.53 ± 1.14	2.66 ± 0.32	4.59 ± 1.54	3748

6	HD	38	F	Soft	+	3.88 ± 1.33	2.75	15.40 ± 2.56	3.05 ± 0.19	4.40 ± 1.84	3749
	Control	33	F	Soft		6.29 ± 2.48	2.42	10.14 ± 1.29	2.47 ± 0.30	3.51 ± 1.28	4384
7	HD	32	M	Hard	...	5.03 ± 1.94	1.71	10.66 ± 1.71	2.60 ± 0.22	2.53 ± 0.97	4256
	Control	32	M	Soft		5.50 ± 1.45	2.40	13.20 ± 1.41	2.77 ± 0.39	2.71 ± 0.63	3908
8	HD	51	F	Soft	...	5.94 ± 3.28	1.14	6.24 ± 0.70	1.91 ± 0.24	1.93 ± 1.91	5168
	Control	53	F			2.96 ± 1.31	2.77	10.39 ± 1.94	2.58 ± 0.22	3.50 ± 1.13	4033
9	HD	58	M	Soft	+	5.89 ± 2.25	2.38	12.87 ± 0.87	2.78 ± 0.26	2.22 ± 0.73	4010
	Control	52	M	Soft		4.61 ± 1.55	1.10	7.40 ± 0.66	2.07 ± 0.28	2.83 ± 0.94	4170
10	HD	56	F	Hard	...	4.46 ± 1.91	1.57	7.80 ± 2.80	2.06 ± 0.21	2.28 ± 0.48	4603
	Control	48	F	Soft		3.00 ± 1.00	1.58	12.07 ± 2.37	2.59 ± 0.39	3.67 ± 0.93	4073
Probabilities‖						(1) NS	NS	NS	NS	NS	NS	NS	NS	NS
						(2) < .05		< .05	< .01	...	< .05	...	< .02	...	< .02

* Biopsies cut into 20 to 24 pieces, 14 to 19 pieces remained attached to dish.

† Arithmetic mean ± SD of 8 to 10 weekly determinations.

‡ Arithmetic mean ± SD of 40 colonies at passage 4 or 5 (same passage for paired cells).

§ Arithmetic mean. One determination at passage 7.

‖ Probabilities for means determined by two-tailed student's *t*-test for paired observations, for variances by *F*-distribution test; (1) HD ≠ control (10 pairs); (2) soft biopsy ≠ hard biopsy (pair 3, 4, 5, 7, and 10); NS = not significant.

blasts have produced collagen, they are mainly characterized by their morphology. A separated fibroblast on a suitable surface moves in a specific direction with the leading edge having many short pseudopodial "ruffles" and its trailing end being

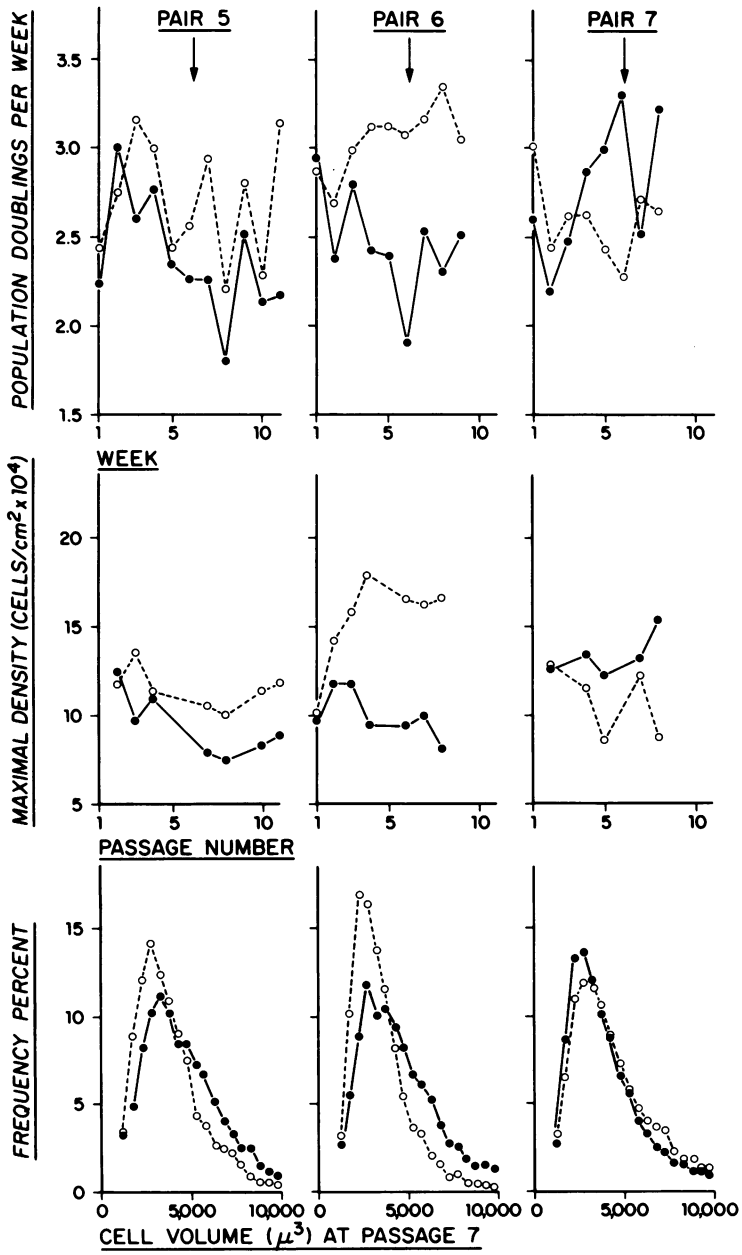

FIG. 1.—Population doublings per week, maximal densities and cell volumes of HD and control fibroblasts; HD (o - - - - - -), control (●———).

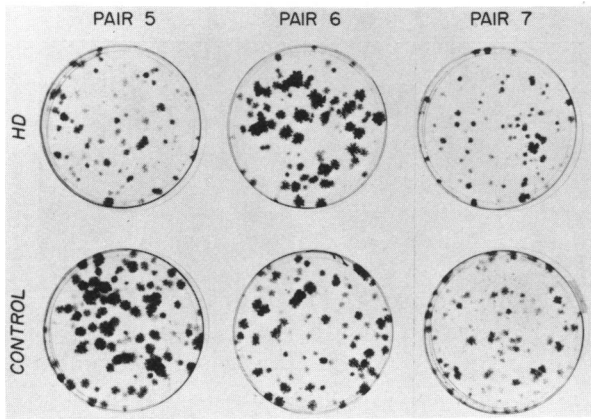

FIG. 2.—HD and control fibroblast colonies 14 days after 250 single cells were placed into each dish.

drawn out into one or several long processes. At low density, fibroblasts form a loose network of interconnected cells, and at high density, they line up next to each other, frequently assuming a spindle shape. Amoebocytes, thought to be derived from blood monocytes, macrophages, or microglia, are less polarized than are fibroblasts, move more randomly, and remain separated. Epitheliocytes, derived from the covering layers of various tissues, form sheets in which the cells are interconnected by desmosomes [10].

It generally has been assumed, as it had been by us, that all fibroblasts grown in vitro from human skin are morphologically and physiologically similar. However, Harper and Grove [11] have shown that fibroblasts from the papillary layer of the human dermis, which is situated just below the epidermis, grow faster and to a higher density than do fibroblasts from the reticular layer that extends from the papillary layer down to the subcutaneous fat. The growth characteristics of a

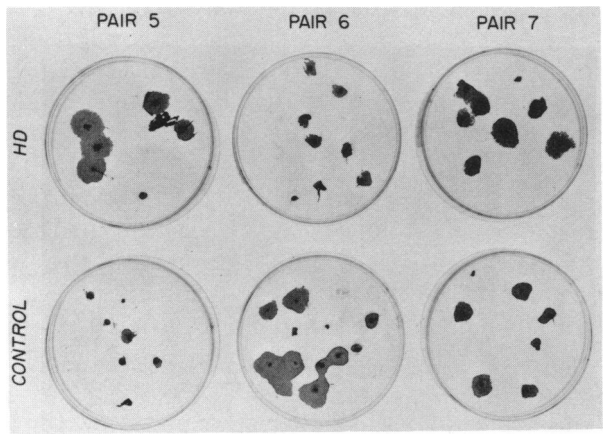

FIG. 3.—Sheets of epithelial cells surrounding pieces of HD or control biopsy tissue at the first culture passage after removal of fibroblasts.

fibroblast culture from a biopsy of the whole thickness of skin, consequently, would depend on the proportion of cells derived from each one of these two layers. Harper and Grove suggest that the reduced life span in vitro of skin fibroblasts from older individuals actually may be due to a lack of papillary fibroblasts, age-related dermal atrophy being more pronounced in the papillary region. Exposure to sunlight exerts deleterious effects, mainly on the papillary cell layer [12]. Cultures from skin with much solar exposure, therefore, would grow slower than cultures from skin with less exposure. Another possibility is that disinfectants and local anesthetics used to prepare biopsy sites may penetrate into the dermis, and, depending on the depth of penetration, differential toxicity might be exerted on papillary and reticular cells resulting in different growth of the cells in culture.

Assuming that a culture of a definite cell type could be obtained reproducibly, one still has to guard against differences in the in vitro age of the cells that are to be compared. Normal human diploid fibroblasts age in vitro showing various metabolic and structural changes [13–15]. We observed that population doublings per week and maximal density gradually decreased, and that cell volume increased. Since the rate of change varied among cell lines, the number of culture passages may *not* correspond closely to in vitro age.

In earlier studies in which we observed significant differences in growth between HD and control fibroblasts, the cells either were grown out directly from biopsies obtained by Dr. Comings in our institution or were obtained from the Repository for Human Genetic Mutant Cells at Camden, New Jersey. The biopsies obtained from our institution were taken from a leg site over the gastrocnemius muscle and grown out under standardized conditions as described in MATERIALS AND METHODS. The cells received from the repository were obtained by different physicians largely from sites on the forearm. Methods employed for disinfection of the skin and anesthesia varied and were not necessarily the same for HD and control biopsies. We recently have learned that some of the HD biopsies that gave rise to the most rapidly growing fibroblast cultures were obtained without the use of a local anesthetic. At the repository, all control cells and some of the HD cells were grown in McCoy's medium, while some of the HD cells were grown in Eagle's MEM. Although we tried to match the ages of donors to within 5 years, in one instance the control was 9 years older than the HD patient. In four of the pairs studied previously, the control cells had gone through one to three more culture passages than did the HD cells at the time we initiated our studies. All of the cells received from the repository had been frozen and thawed at least once prior to our studies, while cells from our institution initially were studied prior to freeze-thaw cycles. A number of studies also were made after recovery from storage. However, recently in our laboratory, we have ruled out the possibility that artifacts might be produced by freezing and storage in liquid nitrogen. Reculture of stored stocks of pairs 2–9 in table 1 showed that those cells in each pair that grew better before freezing did so again after freezing.

A re-evaluation of data now available in our laboratory has shown that for all of the four repository pairs studied the HD member of the pair gave rise to larger colonies from single cells than did the controls. In the case of 10 pairs from the latter

source in which maximal densities and population doublings were measured, eight of the HD samples showed greater proliferative capacity, and two grew to a lesser extent than did the controls. In the case of the pairs obtained in our institution, five HD samples gave larger colonies, and three gave smaller ones; six HD cultures gave greater saturation densities and population doublings, and four gave lower values than did the controls.

During the course of this work, we became aware of a publication by Cassiman et al. [16] who also compared growth in vitro of HD and control skin fibroblasts in a blind study and did not find differences that could discriminate between HD and control cells. These authors caution against comparing cells that were not obtained and grown under identical conditions. We would like to extend precautions to the nature of the skin itself, since fibroblasts from "soft" biopsy tissue appeared to grow better than those from "hard" tissue. There are regional differences in the structure of the skin and changes due to age, disease, and exposure to sunlight or chemicals. We now feel that structural differences in the skin might lead to cellular differences in vitro even if conditions for obtaining the biopsy and culturing the cells are standardized. We would like to suggest that a histological examination should be performed for each biopsy explanted, and possibly, different cell layers should be dissected out and grown separately.

Heeding the above stated precautions and modifying our protocol accordingly, we are proceeding to continue our comparisons of HD and control fibroblasts with regard to affinities for cholinergic, GABAergic, and noradrenergic ligands and insulin, and epidermal and fibroblast growth factors. In addition, studies are being carried out to determine whether or not differential effects are exerted on growth and metabolism of HD and control cells by variations in temperature and oxygen tension. Compositions of intracellular amino acid pools and lipid distributions also are being studied.

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