Insulin-dependent Childhood Diabetes

Normal Viability of Cultured Fibroblasts

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SUMMARY

Cultured skin fibroblasts from clinically normal offspring of two parents with non-insulin-dependent diabetes have demonstrated premature senescence as a decreased ability of cells to establish colonies when inoculated at low density (plating efficiency). The present study tested the hypothesis that there is an inherent cellular defect affecting viability of diabetic cells in insulin-dependent diabetes. Four insulin-dependent patients, aged 12 to 19 years, included two with joint contracture, skin changes, and growth failure; one with thyroiditis and past history of nephrosis; and one with a family history of insulin dependency. Ten control subjects, aged 10 to 52 years, had negative family histories and normal oral glucose tolerance tests.

Number of cells per confluent dish correlated significantly with donor age ($p < 0.001$) at 30 and 40 in-vitro generations. The patients' cells' mean confluent density did not differ from that of five age-matched controls. Plating efficiency correlated with donor age at 30 in-vitro generations ($p < 0.001$); plating efficiency of cells from the youngsters with diabetes was virtually identical to that of control cells at 20, 30, and 40 generations.

In this small series of two subjects with in-vivo growth failure, one with associated autoimmune disease and another with familial insulin-dependent disease, cultured fibroblasts demonstrated normal viability and the hypothesis of a cellular growth defect was not confirmed. DIABETES *27*:338-41, March, 1978.

Fibroblasts cultured from skin biopsies faithfully reproduce genetically determined donor characteristics.[1-4] This fidelity has been thought to include performance of the donor's inherent program of biologic aging; in-vitro life span of cultured fibroblasts correlates inversely with the chronologic age of the cell donor.[5,6] The total number of cells attained at confluency also correlates inversely with donor age.[7] Cultured fibroblasts from clinically nondiabetic offspring of two parents having non-insulin-dependent diabetes have demonstrated premature senescence as a decreased ability of cells to establish colonies when inoculated at low density (plating efficiency).[8] This evidence of genetically programed accelerated senescence of tissues in diabetes mellitus has formed the basis for hypotheses to explain the diabetes-associated endocrine, connective tissue, and vascular pathology.[9-12]

The purpose of this study was to test the hypothesis, in cultured fibroblasts from young patients with insulin-dependent diabetes mellitus, that there is an inherent defect in viability of diabetic cells.

MATERIALS AND METHODS

Skin biopsies were obtained from 10 healthy persons aged 10 to 52 years with no known family history of diabetes mellitus and normal four-hour glucose tolerance tests. Biopsies from four patients with insulin-dependent, ketosis-prone diabetes mellitus were handled in an identical manner to those from controls. The patients (table 1) were a 12½-year-old boy with diabetes for 4½ years associated with growth delay, multiple mild joint contracture, and thick, tight, waxy skin;[12] an 11½-year-old boy with a 10-year history of diabetes and evidence of associated autoimmune disease; a 15-year-old boy biopsied at the time of diagnosis (with classic symptoms, hyperglycemia and ketonuria) who had three second-degree relatives with insulin-dependent diabetes; and a 19-year-old girl with growth failure, sexual infantilism, multiple small and large joint limitation, skin changes, and proteinuria after 12 years of diabetes.[12] None of the patients had ever had prolonged difficulty with control of their diabetes or been hospitalized for diabetes after the diagnosis and initial treatment.

Biopsies were 3-to-4-mm.-wide partial dermal excisions from the volar surface of the nondominant

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TABLE 1

Clinical features of patients with diabetes mellitus

	1	2	3	4
Age at biopsy (yr.)	15-3/12	11-6/12	12-5/12	19
Sex	M	M	M	F
Age onset of diabetes (yr.)	15-3/12	1-11/12	8-5/12	7
Duration insulin therapy (yr.)	—	9-7/12	4	12
Insulin dose (U./kg./day)	0.31 (at 16 yr.)	0.40	0.57	0.38
Height age	Tall adult	8	9-10/12	12-10/12
Change height age* / Change chronologic age	—	0.6†	0.58	0.75
Other	Family history of insulin-dependent diabetes	Nephrosis at age 6; Hypothyroidism detected at age 12	Mild finger contracture and tight skin	Proliferative retinopathy at age 19-6/12; multiple joint and skin involvement

*Over previous year.
†Increased to 1.2 on thyroid replacement with poor compliance.

forearm. The tissue was dropped into a vial containing growth medium (Eagle's essential medium with 15 per cent fetal calf serum) and transferred to a laminar flow hood for immediate explanation. The tissue was diced into 20 to 30 pieces with scalpels. Three fragments were transferred in a drop of medium to the center of each of eight to 10, 60×15-mm. tissue culture Petri dishes. A dab of sterile stopcock grease was applied to each dish, and a 25-mm.^{2} cover slip was pressed in place so that one corner was held to the dish and the fragments of tissue were sandwiched under the glass. Growth medium (5 cc.) was added and the explants incubated at 36 to 37° C. in 5 per cent carbon dioxide/95 per cent air. The medium was changed every seven days; cells were ready for subculture at the end of three to four weeks. Medium was removed and the cells were released in 1 cc. of 0.125 per cent trypsin, transferred to 60×15-mm. dishes, and reincubated in 5 cc. medium to confluency. They were then subcultured at 1:2 dilution and, at confluency, subcultured at 1:4 dilution. In another week to 10 days they were subcultured at 1:8 dilution and were then carried on through successive subcultures at 1:8 dilutions. No antibiotics were used. Several cultures of randomly chosen cells at the end of the study failed to reveal Mycoplasma contamination.[13] In addition, most strains were cultured in Lab-Tek chambers and stained (aceto-orcein) for Mycoplasma, but no organisms were found.[14] The same lot of fetal calf serum was used for the entire study.

At the time of initial subculture the fibroblasts were thought to be at 10 generations (doublings). A 1:2 split requires one doubling, 1:4 two, and 1:8 three generations to reach confluency.

At 20, 30, 40, and, in some cases, 50 generations, two confluent plates of each strain were released by trypsin and cell counts carried out on a hemocytometer. Trypsinized cells were diluted with medium to give a concentration of 1,000 per cubic centimeter; 0.5 cc. was then inoculated into each of four dishes containing 5 ml. growth medium. The medium was replaced on day 7; on day 14 the medium was removed and the cells stained with Giemsa. Macroscopic colonies were counted to provide an estimate of plating efficiency (100 × mean number of colonies per dish ÷ 500).[8]

RESULTS

No significant age differences were noted in time for fibroblast outgrowth to occur from explants or time from subcultures to confluency. Cells from the patients with diabetes were identical to the others in these measures.

Confluent density (figure 1) was correlated to age with significance ($p < 0.001$) at 30 and 40 ($r = 0.86, 0.90$) but not at 20 generations ($r = 0.13$). The cells from five young controls did not differ from those of the four patients in this measure (at 20 generations, controls $1.8 \pm 0.2 \times 10^6$ cells per plate vs. patients 2.5 ± 1.5 S.D.; at 30, 1.8 ± 0.3 vs. 1.8 ± 0.6; and at 40, 1.3 ± 0.1 vs. 1.5 ± 0.4).

Plating efficiency was significantly correlated with donor age only at 30 ($p < 0.001$) but not at 20 or 40 generations (figure 2). Plating efficiency of cells from youngsters with diabetes was virtually identical to that of control cells at 20, 30, and 40 generations (figure 3).

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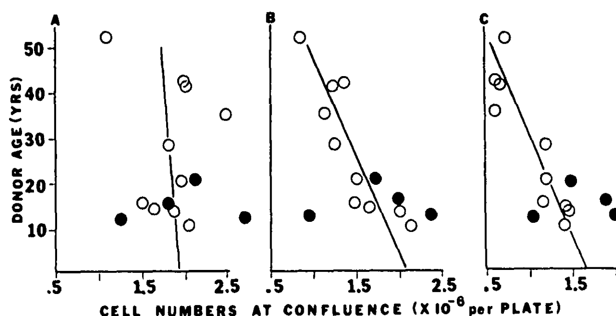

FIG. 1. Cell numbers per confluent dish (60 mm.) according to donor chronologic age at in-vitro generations 20(A), 30(B), and 40(C). Each symbol is the mean of two dishes, with variability between paired counts not greater than 10 per cent. Open circles represent cells from normal subjects, closed circles cells from patients with diabetes. The regression lines, based on the control data, are significant for B (r = 0.86) and C (r = 0.90) but not for A (r = 0.13).

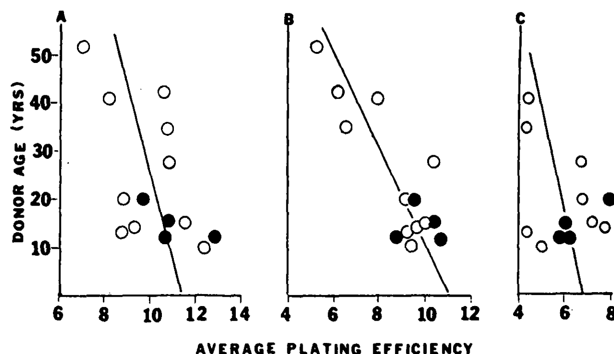

FIG. 2. Plating efficiency of cultured fibroblasts according to donor chronologic age at in-vitro generations 20(A), 30(B), and 40(C). Each symbol is the mean of four dishes; open circles represent cells from control subjects, closed circles cells from patients with diabetes. The regression lines, based on control cells only, are significant in B ($r = 0.84$, $p < 0.001$) but not in A (r = 0.49) or C (r = 0.35).

DISCUSSION

This study confirms the inverse correlation of total cell numbers at confluency with age of the donor.[7] High correlation for this presumed in-vitro measure of biologic aging at 30 and 40 in-vitro generations indicated greater predictability than did plating efficiency. The latter was correlated significantly with the age of donor only at 30 in-vitro generations. Cells from the youngsters with diabetes attained normal confluent density, failing to reflect the severe in-vivo growth defects present in two of them.

Plating efficiency is a measure of the ability of individual cells or clones to attach and form colonies. This reflects differences in viability that might be obscured in mass culture, in which cell interaction occurs whereby weaker cells might be assisted by more hardy neighbors.[8] Decrease in plating efficiency occurs during in-vitro aging, before mass cultures show evidence of decline.[8,15]

Failure to demonstrate a difference of plating efficiency between normal and diabetic fibroblasts in this study contrasts with the findings of Goldstein et al.[8] in clinically normal subjects who were presumed to be at risk for diabetes (offspring of two parents with overt diabetes). Our method is similar to that of Goldstein, and the mean efficiencies at each decennary generation are similar. We have shown significant donor-age inverse correlation with plating efficiency at mid-passage (30 generations), which emphasizes the sensitivity of this measure of cell viability as a reflection of the biologic aging program of the donor.[8,15]

Our expectation, therefore, was to demonstrate in-vitro premature senescence in the youngsters with diabetes, and to that end a biased population was studied. Two of the subjects had in-vivo growth failure and maturational delay, with changes in the periarticular connective tissues and integument resulting in limitation of joint mobility and thick, tough

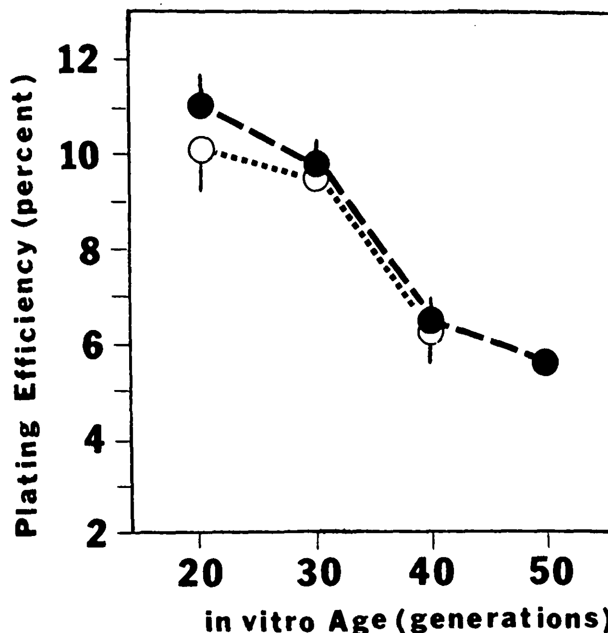

FIG. 3. Plating efficiency of cultured fibroblasts from five control subjects 10 to 20 years of age (open symbols) and four children 12 to 19 years old with insulin-dependent diabetes mellitus (solid symbols) at decennary in-vitro generations. Each point represents the mean of average efficiencies for each donor. Vertical bars are standard error of the mean and where not shown are encompassed by the symbol. Only patient data were available at generation 50 and were shown to indicate persistent viability.

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skin;[12] one of these patients (table 1, no. 4) has subsequently developed proliferative retinopathy. One had two other disorders of immune aggression, a circumstance thought to accelerate morbidity and mortality in diabetes,[16] and the new patient had a remarkable family history for insulin-dependent diabetes. Nonetheless, the patient group's cells demonstrated unequivocally normal viability in vitro and failed to confirm the hypothesis that these youngsters have a cellular growth defect. More subtle expressions of the diabetic genotype in relation to aging phenomena might be found in the production of specific proteins by the cultured cells, such as HLA antigens,[17] enzymes,[18] and insulin receptors.[19]

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