Diabetes Mellitus and Genetic Prediabetes

DECREASED REPLICATIVE CAPACITY OF CULTURED SKIN FIBROBLASTS

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ABSTRACT The idea that the gene(s) that cause diabetes mellitus can be expressed in extrapancreatic cells has been examined by tissue culture techniques. Skin biopsies were obtained from 25 normal subjects (N), 26 overt diabetics (D), 16 of juvenile onset (JOD) and 9 of maturity onset (MOD), and 21 subjects genetically predisposed to diabetes (P) on the basis of maturity-onset diabetes in both parents. Each biopsy was subdivided, multiple skin fragments were explanted in vitro, and several parameters of cellular outgrowth were monitored in primary and secondary cultures until cell division ceased because of senescence. In general, the rank order of growth vigor was N > P > D although differences were often marginal and statistically significant between N and JOD and(or) MOD. Outgrowth of epithelial cells was more vigorous in N explants in early stages, but later, JOD and MOD cells grew better than those of N. Outgrowth of fibroblast cells from N explants was more vigorous both at early and later stages and required less time to achieve maximum percent outgrowth. In secondary cultures, N cells grew faster than the other three groups so that fewer days elapsed between subcultures but significant differences were only seen between N and one or two of the other groups over some of the first seven subcultures. The onset of cellular senescence occurred earlier in P and JOD cultures both in mean population doublings and calendar time. N cultures had a higher percent surviving clones after picking than MOD, and a shorter recloning time than clones of JOD. The replicative life-spans of cultures (mean population doublings ±SE) were N = 52.54±2.24, P = 47.84±2.43, JOD = 47.12±2.99, and MOD = 46.40±4.04, but differences did not reach significance for N vs the other three groups.

The data demonstrate that cellular growth is impaired in both JOD and MOD types of cultures and to a generally lesser extent in P cultures. This is consistent with intrinsic genetic defects but the possibility that persistent deleterious effects of in vivo pathophysiology contribute alone or in combination cannot be ruled out. Therefore, the diabetic defect(s) can be expressed in extrapancreatic cells of mesenchymal origin. This system should prove useful in exploring the interplay between genetic and environmental factors in diabetes, the mechanisms(s) of hyperglycemia and other metabolic derangements, and the propensity that affected individuals have to develop degenerative diseases.

INTRODUCTION

Diabetes mellitus is a complex disorder of unknown etiology (1). Whereas the potential to develop diabetes is inherited, the precise nature of genetic transmission remains a mystery (2–4). Recent studies indicate that great heterogeneity exists in the clinical and pathological picture, and it follows that the genetic basis will be equally diverse (2–4). Moreover, like many complex genetic disorders, the variable expression of diabetes is influenced by several environmental factors such as viruses, nutrition, obesity, and in particular, the aging process (1–5).

Irrespective of the underlying basis and provocative agents, clinical expression occurs when there is an absolute or relative deficiency of effective insulin leading to an inappropriately high level of blood glucose. But whereas this definition implicates endocrine cells

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that secrete hormones involved in glucose metabolism, it is now clear that certain specific forms of diabetes have defects that reside in nonendocrine tissues. Thus, despite the delivery of active insulin, often in abnormally high concentrations, these "peripheral" cells show a blunted response (6–11). Because the biochemical components of cells are genetically determined, and many gene products are expressed in all cells, some defects may be shared such that both hormone production and peripheral responsiveness are simultaneously defective (12, 13).

In an earlier study we demonstrated that skin fibroblasts cultured from donors genetically predisposed to diabetes had reduced growth capacity compared to normal controls, showing a reduced ability to form colonies (14). Such "prediabetic" donors were used rather than overt diabetics to preclude any adverse effects on in vitro growth that could ensue as a result of prior in vivo derangements of metabolism or anatomic pathology. In the present report we have examined a new series of normal subjects and individuals genetically at risk to develop diabetes. We have also added overt diabetics with both juvenile-onset and maturity-onset forms of the disease who were predominantly free of clinical sequelae. The results to be presented confirm and extend the previous findings on the deleterious effects of the diabetic genotype and(or) metabolism on cellular growth in vitro. Preliminary reports have been published (15–17).

METHODS

Subjects

The normal group. This group consisted of subjects in excellent health with repeatedly normal glucose tolerance tests and a negative family history of diabetes (Table I).

The high-risk (prediabetic) group. Subjects in this group were offspring of two parents with clinically overt maturity-onset diabetes (Table I). These subjects will be referred to as high risk or prediabetic even though it has been reported that only 35% will go on to develop overt diabetes by age 85 yr (18). After the first analysis (15) a group of nine prediabetics was eliminated from the series. These subjects either had one parent with juvenile-onset diabetes (three subjects), were the nondiabetic monozygotic twin of an overt diabetic (four subjects), or were offspring of parents with insufficiently documented diabetes (two subjects). The first seven were excluded because their risk to diabetes seems to represent an entirely different entity (19–22).

TABLE I

*Age and Sex of Subjects and Percent Ideal Body Weight at Time of Biopsy**

	n	Sex M	Sex F	Age	Age range	Weight‡
				yr	yr	%
Normal	25	8	17	44.3±17.5	15–76	104.6±14.6
Prediabetic	21	13	8	40.0±15.1	19–62	108.9±24.5
Juvenile-onset diabetes	16	8	8	29.3±3.0	14–60	98.0±13.8
Maturity-onset diabetes	9	6	3	59.7±13.4	39–76	107.9±4.0

* Mean±SD.

‡ From Metropolitan Life Insurance Tables (1959) based on body build and height.

Each prediabetic had received at least two standard 3-h oral glucose tolerance tests (18), the last one within 1–5 mo before biopsy. The high-risk subjects had predominantly normal criteria throughout these tests. A few had "chemical diabetes" with one or more abnormal blood glucose levels after an oral glucose challenge, but none had fasting hyperglycemia.

The juvenile-onset and maturity-onset diabetic groups. These groups (Tables I and II) consisted of individuals with overt diabetes who were outpatients or ambulatory inpatients in the Diabetes Teaching Unit of the Joslin Clinic.

Juvenile-onset diabetes was defined, for 15 of the 16 subjects in this study group, as the development of continuous insulin requirement with onset under 19 yr of age. The remaining subject had onset at 31 yr of age, but required multiple daily insulin doses for adequate control. All maturity-onset diabetics were 36 yr of age or older at onset. Most subjects were free of renal, cardiovascular, and ocular manifestations of diabetes (Table III).

Biopsy and preparation of tissue

Biopsies were scheduled in three sessions about 8 wk apart at the Joslin Research Laboratory in Boston. Subjects from the four study groups were assigned consecutive code numbers as they appeared in random order for biopsy. After biopsy, skin samples were immediately placed into regular growth medium (*vide infra*) and kept at 0–4°C. All preparations were then transported from Boston to Hamilton and explanted within 48±2 h in the same sequence as biopsied. All tissue culture operations were carried out by one of us (E. J. Moerman) with no knowledge of the specific identities or group categories of each subject.

Biopsy techniques have been previously described (14, 23, 24). In brief, a 4-mm diameter plug of skin was excised with a stainless steel punch and scissors from the anterior forearm 1–2 in below the elbow crease with Xylocaine anaesthesia without epinephrine (Astra Pharmaceutical Products, Inc., Worcester, Mass.). A full thickness one-quarter portion of the biopsy was divided with sharp scalpel blades, prepared for frozen section, multiply sliced in 2 μm thickness with a microtome, mounted on slides, stained by hematoxylin and eosin, then stored for later histological examination.

Another one-quarter portion was similarly removed for direct dissociation of cells. This protocol was carried out on the first 25 subjects in an attempt to determine the primary plating efficiency of cells dissociated directly from skin. The procedure involved mincing of skin with fine scissors followed by sequential incubation with trypsin and DNase, and filtering through a sterile nylon mesh of 55 μm pore size. Cells in the resulting filtrate were counted and 1,000–5,000 cells were plated into 100 × 20-mm (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) Petri dishes. Attempts were also made to separate epidermis from dermis, (the latter being the precursor layer for cultured fibroblasts) to reduce the number of "background" epidermal cells, which would not contribute to colony formation under these conditions. Skin fragments were digested with collagenase and the epidermal

TABLE II
*Clinical Details on Diabetic Subjects**

Parameter	Juvenile onset		Maturity onset	
Age at onset, *yr*	14.2±5.9		48.2±8.4	
Mean duration of diabetes, *yr*	15.1		11.4	
Symptoms at onset				
Acute (polydipsia, polyphagia, polyuria)	12		5	
Mild	3		3	
None	1		1	
Birth weight, *pounds*	6.8±1.9	(n = 11)‡	6.9±2.6	(n = 5)
Parents with diabetes				
None	13	(n = 14)	6	
One	1		3	
Number of diabetic siblings		(n = 15)		(n = 8)
None	14		4	
One	0		2	
Two	1		0	
Three	0		2	
Number of siblings	2.3±1.6		5.3±5.4	
Number of diabetic children	0	(n = 15)	0.1±0.3	
Number of children	1.1±1.4		2.2±0.9	
Percent ideal weight at onset, %	90.0±30.1 (12)		122.7±11.8	
Initial treatment				
Diet	1		1	
Oral agent	1		4	
Insulin	14		4	
Current treatment				
Oral agent	0		2	
Insulin	16		7	
Insulin dose				
Single	7		4	
Split	9		3	
Units of insulin per day, *U*	43.6±22.7		37.8±11.2	

* Mean±SD.

‡ Where $n < 16$ for juvenile-onset diabetes or <9 for maturity-onset diabetes, data were not obtainable.

layers removed with forceps under a dissecting microscope. The remaining dermis was dispersed and the cells plated. Extremely erratic results led us to abandon this portion of the protocol.

The remaining portion of the skin biopsy was subdivided with sharp scalpel blades into ≅1-mm full thickness fragments. Three such fragments were explanted into each 60 × 15-mm Petri dish where they were evenly spaced and pressed under a 25-mm² glass coverslip secured at one corner by a pat of sterile silicone grease (14, 23, 24). In the first group of biopsies, where one-quarter of the material was consumed in attempts at direct dissociation of skin, 12 skin fragments (i.e., four replicate dishes) were explanted. In subsequent groups enough skin was available for explantation so that six–eight replicate dishes were used (i.e., 18–24 skin fragments) from each donor.

Medium

Eagle's minimum essential growth medium supplemented with "non-essential" amino acids, Fe $(NO_3)_3$, pyruvate, and 250 mg/dl glucose was used throughout the study (23). Fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) was added to 15% by volume. Limited space for frozen storage necessitated the purchase of serum from two lot numbers. The order of biopsies and subsequent cultures was randomized among the four study groups to adjust for possible systematic bias in growth performance caused by different serum lots. In addition, when several cell strains developed in medium that contained one lot of serum were cultivated in medium that contained the second lot, growth parameters appeared almost identical.

Incubation of explants and secondary cultures

All tissue culture material was incubated at 37.0±0.3°C in a humidified atmosphere of 95% air and 5% CO_2, with total medium replacement once each week. Skin fragments were scored every 2 d for various growth parameters (Fig. 1). The growth of explanted tissue in culture is known as the "primary culture" stage, whereas that after harvest of cells produced by explants is known as "secondary culture." A complete glossary of terms used in this study protocol appears in the Appendix. The time of harvest was determined by the appearance of the first confluent fibroblast sheet in explant dishes. The two dishes of explants showing the best growth were chosen for

TABLE III

Presence of Pathophysiological Sequelae in Diabetic Groups

Type of sequelae	Juvenile-onset diabetes		Maturity-onset diabetes	
	Present	Absent	Present	Absent
Cataracts	1	15	0	9
Ocular hemorrhages	1	15	2	7
Microaneurysms	3	13	2	7
Exudates	3	13	4	5
Neurological (paraesthesiae and so forth)	6	10	5	4
Claudication	0	16	2	7
Gangrene	0	16	0	9
Amputation	0	16	0	9
One or more nonpalpable pedal pulses	0	16	1	8
Stroke	0	16	0	9
Congestive heart failure—treated	0	16	2	7
Angina	0	16	2	7
Myocardial infarction	1	15	2	7
Cardiac arrhythmia	1	15	2	7
Proteinuria	1	15	1	8
BUN >25 mg/100 mg	1	8 (n = 9)	1	6 (n = 7)
Triopathy*	2	14	1	7 (n = 8)

* Triopathy indicates simultaneous: (*a*) ocular changes in the lens and(or) retina i.e. hemorrhages, exudates, and microaneurisms. (*b*) renal impairment as persistent proteinuria and(or) elevation of blood urea nitrogen (BUN) and creatinine, and (*c*) neurological (sensory-motor) impairment by objective clinical criteria.

secondary culture. In most cases all three fragments within a dish, and never fewer than two, showed the requisite amount of growth. For harvest, cells from both dishes were liberated and pooled after treatment with a 0.125% solution of trypsin (Difco Laboratories, Detroit, Mich., 1:250) in phosphate-buffered saline for 5–10 min at 37°C. After addition of 10 vol of growth medium to inhibit enzyme action, cells were dispersed by rapid pipetting. An aliquot of the cell suspension was counted on a Cytograf electronic cell counter (25), which was frequently calibrated with hand counting on a hemocytometer. Cells were inoculated at low density (500 per dish) into six replicate 100 × 20-mm plastic Petri dishes. The remaining cells were inoculated into two 100 × 20-mm Petri dishes at high density for continuous passage at a 1:4 subculture ratio. This expression indicates that the harvested cells were diluted 1:4 with respect to the surface area of the 100-mm dish. Thus, when cells became confluent, 2 mean population doublings (MPD)[1] were counted 14, 26). The number of MPD that occur in attaining the first confluent monolayer in primary cultures is unknown. No attempt has been made in this study to estimate this value, and therefore, it has not been included in calculations of MPD.

When cells in each of the secondary culture dishes became confluent, they were subdivided at a 1:4 dilution ratio into four dishes with identical surface areas, again counting 2 MPD when confluency was achieved. Counts were not performed routinely to quantify the percent of cells that adhered after each subculture. However, this measurement on randomly selected cultures ranged from 80 to 100% for early passage cultures to as low as 50% at late passage with no significant differences between groups. For simplicity, therefore, a 100% plating efficiency was assumed in determining the number of MPD required to achieve confluence between subcultures. Although this underestimates the cumulative number of MPD in all cases, the four groups can still be compared with validity.

Confluent cells harvested after the second subculture, i.e. at 4 MPD, were banked in multiple aliquots in liquid nitrogen in sealed glass ampules that contained 0.5 ml of cells (1-2 × 10^6 cells/ml) in growth medium plus 10% dimethyl sulfoxide. In general, the remainder of the study was carried out in 60-mm dishes with serially passaged cells that were uninterrupted by storage in liquid nitrogen. In a few cases where strains were lost because of sporadic contamination, cells were reconstituted from frozen storage at 4 MPD and subcultivation was resumed from that stage. Good agreement was found in various growth parameters, such as low density plating efficiency and growth rates of mass cultures, between the continuously cultivated cultures before contamination and cells newly reconstituted from frozen storage.

After MPD 4, all subcultivations were carried out at 1:8 dilutions counting 3 MPD with each such manipulation. All dishes were inspected daily by microscope and passaged within 1 d of becoming confluent. At early passage, most cell strains became confluent <7 d after subculture. Cells that failed to reach confluence within this time were refed with fresh growth medium. Toward the end of their life-spans, cells grew slower and became swollen and granular in spite of weekly medium renewal.

Low density plating efficiency was determined at the nearest decile of MPD, i.e., at harvest time (0 MPD), 10, 19, 31, 40, and 49 MPD by diluting a known concentration of suspended cells with growth medium. Suspensions of 250–1,000 cells were inoculated into dishes of 60 or 100 mm diameter. Cells were refed 1 wk later and after 2 wk dishes were stained with Giemsa and scored for plating efficiency. Replicate dishes that contained cells plated at 0 MPD were examined by transillumination to identify mature colonies, which were circled with a red wax pencil. The circled colonies were then examined microscopically under low power (×40), and the margins were precisely delineated. Five colonies were carefully selected as distinct from neighboring colonies and were then "picked" by the steel cylinder method of Puck et al. (27). Briefly this method involved aspiration of the medium, rinsing, and then placing a steel cylinder (height 12 mm, o.d. 9 mm, i.d. 6 mm) over a colony and securing it with sterile silicone grease. Cells were liberated by adding three or four drops of trypsin solution into the cylinder, then flooding the remainder of the dish with growth medium, and incubating for 5–10 min at 37°C. Several drops of medium were then added to the interior of the cylinder to arrest trypsin action. The cells were removed with a pasteur pipette, transferred to a 35-mm dish, and incubation continued under standard growth conditions. Colonies that produced confluent sheets were then replated after counting and dilution, followed by Giemsa staining and scoring as secondary colonies.

[1] *Abbreviations used in this paper*: JOD, juvenile-onset diabetes; MOD, maturity-onset diabetes; MPD, mean population doubling(s).

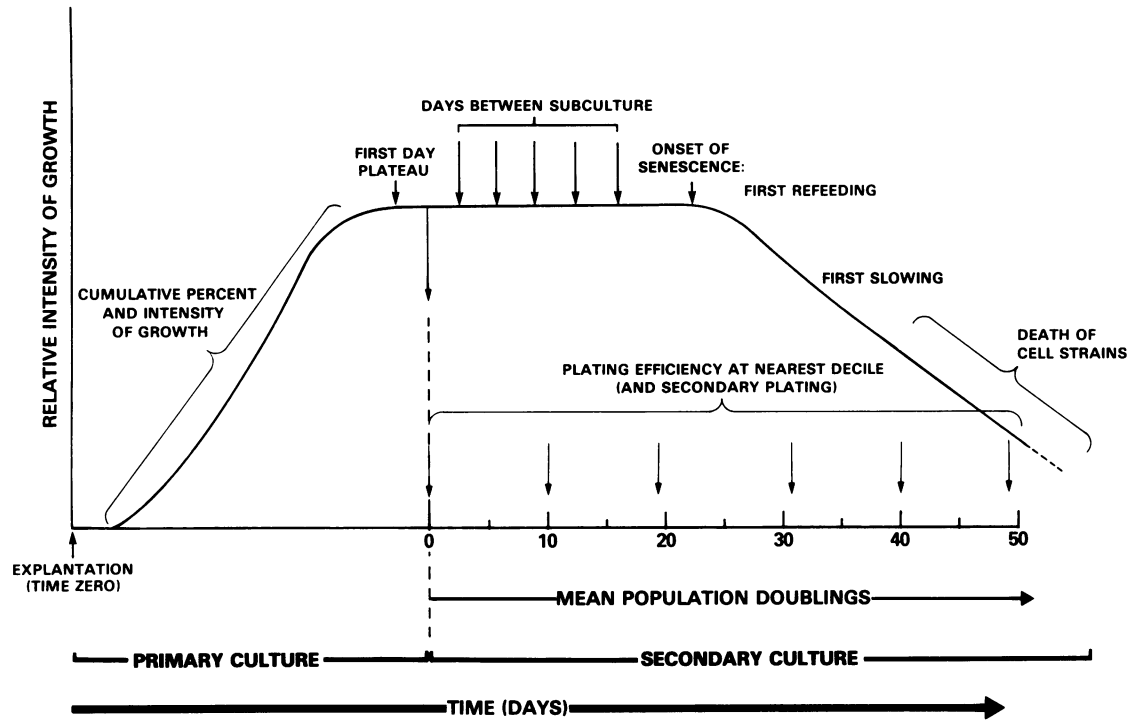

FIGURE 1 Diagram illustrating parameters used to monitor vigor of cellular outgrowth from diabetic, prediabetic, and normal skin explants and subsequent secondary cultures. Harvest day is the time of the first subculture and is designated as 0 MPD. Determination of low density plating efficiency begins at this stage and continues at the nearest decile of MPD (see text).

Dr. Leonard Hayflick of Stanford University Medical School examined all cell strains at least twice during their life-span and again on termination for mycoplasma with negative results.

Analysis of data

On completion of the tissue culture portion of the study, data were transcribed to precoded forms, keypunched, and analyzed on an IBM 370/168 computer (IBM Corp., White Plains, N. Y.).

The null hypothesis proposed for this study was that fibroblasts from normal control subjects would perform no better in tissue culture than those obtained from the offspring of two diabetic parents, juvenile-onset diabetics, or maturity-onset diabetics. The decision to accept or reject this hypothesis was based upon a single-tailed t test procedure (*vide infra*).

Preliminary analyses indicated that the mean ages among the study groups differed significantly. Because donor age is a critical factor in the performance of fibroblasts in tissue culture (12–14, 26, 28), the analysis of covariance was used to adjust for these age differences (29). The adjusted means for normal controls were then compared with those from the other three groups in all the data presented below. The standard Student's t test, however, is not valid for such comparisons, because they are not independent. Therefore Dunnett's one-sided t test for comparing all means with a control (30) was used. Conventional Pearson's correlations were used to determine if significant relationships existed between variables within the study groups (31).

RESULTS

Primary Culture

Epithelial cells. Epithelial cells from explants of normal donors showed an early advantage at day 2 and 4 in intensity of growth per fragment (Table IV). However, juvenile-onset diabetes (JOD) cells as well as prediabetic cells appeared to outgrow normal cells at later stages from day 10 to day 14, after which involution began to appear. There were no significant differences between the four groups in the percent of fragments showing epithelial growth (not shown). It was noteworthy that by the 1st day of observation (day 2), over 60% of the fragments in all four groups were positive for growth with the maximum only rising thereafter to 80–87% on day 14. The 1st plateau in normals occurred at 5.82±0.70 d (mean±SE) compared to 6.20±0.76 in prediabetics, 7.64±0.93 in JOD, and 7.00±1.26 d in maturity-onset diabetes (MOD), but the differences between these means were not statistically significant.

Fibroblasts. Unlike epithelial cells, normal fibroblasts performed significantly better than JOD in the percent of fragments showing growth, but only at the single early stage of day 4 (Table V). The mean maximum percent of fragments showing growth was about

TABLE IV
*Primary Culture: Growth of Epithelial Cells Per Fragment: Cumulative Score**

Day	Normal	Prediabetic	Juvenile-onset diabetes	Maturity-onset diabetes	*P* value‡
2	1.22±0.09	1.00±0.10	0.75±0.12	0.78±0.16	N vs. JOD < 0.01; N vs. MOD < 0.05
4	1.78±0.08	1.66±0.08	1.43±0.10	1.48±0.14	N vs. JOD < 0.01
6	1.96±0.09	1.92±0.10	1.82±0.12	1.89±0.16	NS
8	2.17±0.11	2.32±0.12	2.48±0.15	2.28±0.20	NS
10	2.55±0.14	2.93±0.15	3.33±0.19	2.66±0.26	N vs. JOD < 0.01
12	2.87±0.18	3.44±0.20	4.05±0.25	3.37±0.33	N vs. P = 0.05; N vs. JOD < 0.01
14	3.10±0.26	4.17±0.28	5.17±0.34	3.92±0.46	N vs. P < 0.01; N vs. JOD < 0.01

Epithelial cells were scored with each unit representing an outgrowth radius of 0.24 mm. The maximum possible score per fragment was 10 U (see Appendix for further details).
* Mean±SE arbitrary units.
‡ N, normal; P, prediabetic; JOD, juvenile-onset diabetes; MOD, maturity-onset diabetes.

72–79% with no significant differences between the four groups. A large amount of variation was found in each group at all stages. In terms of the cumulative vigor of outgrowth per fragment (Table VI), there was significantly better growth in normal than in both JOD and MOD explants at day 4, and again at days 12–16 between normals and JOD. Thereafter all four group scores became similar, but unlike the case in epithelial cells, none surpassed normals at later stages. The 1st d plateau was attained earlier in normals compared to prediabetics, JOD, and MOD (Table VII).

The day of harvest ranged from 19 to 38 d after explantation of skin fragments for all study groups combined with an overall mean of 24.85 d. There were no significant differences between groups for this parameter.

Secondary cultures

Normal fibroblasts appeared to grow more vigorously requiring fewer days to attain confluence (Fig. 2) but significant differences were only noted between normals and one or two of the other three groups at some of the first seven subcultures. Although the growth advantage of normals appeared to persist over this time, all four groups showed a gradual lengthening of the growth interval up to nine subcultures (not shown). After this time, a progressive number of cell strains ceased to divide resulting in a decline in the number of cultures and an increase in variability with no further significant differences observed.

Senescence. The onset of senescence appeared significantly earlier in prediabetic and JOD cells such that refeeding was required earlier than in normal strains (Table VIII). This was seen in terms of MPD level but not for calendar time. The apparent differences between normals and MOD did not reach significance because of the larger variance in the latter group. The number of MPD and days to slowing showed similar trends between normals and the other groups

TABLE V
*Primary Culture: Percent of Fragments Showing Fibroblast Growth**

Day	Normal	Prediabetic	Juvenile-onset diabetes	Maturity-onset diabetes	*P* value
			%		
2	0.35±0.27	0.02±0.29	0.36±0.36	0.21±0.49	NS
4	17.36±3.08	16.97±3.34	5.90±4.10	6.00±5.55	N vs. JOD < 0.05
6	32.94±3.48	38.37±3.79	32.56±4.65	28.10±6.29	NS
8	45.03±3.80	51.10±4.13	47.31±5.07	40.24±6.86	NS
10	58.01±3.12	59.78±3.39	57.54±4.16	55.87±5.63	NS
12	65.43±2.78	68.12±3.02	65.92±3.70	61.64±5.01	NS
14	70.38±2.83	72.87±3.07	69.38±3.77	66.68±5.10	NS
16	70.92±2.85	74.14±3.10	72.18±3.80	70.91±5.14	NS
18	71.43±2.81	76.05±3.05	75.15±3.75	75.42±5.07	NS
20	71.88±2.71	78.24±2.95	78.12±3.62	78.80±4.90	NS

Abbreviations as in Table IV.
* Mean±SE.

TABLE VI
*Primary Culture: Fibroblast Growth Per Fragment: Cumulative Score**

Day	Normal	Prediabetic	Juvenile-onset diabetes	Maturity-onset diabetes	*P* value
2	0.007±0.003	0.0003±0.004	0.002±0.004	0.004±0.006	NS
4	0.19±0.03	0.16±0.03	0.05±0.04	0.07±0.05	N vs. JOD < 0.01; N vs. MOD < 0.05
6	0.34±0.04	0.39±0.04	0.34±0.05	0.28±0.06	NS
8	0.56±0.05	0.60±0.06	0.54±0.07	0.49±0.09	NS
10	0.93±0.07	0.87±0.07	0.80±0.09	0.82±0.12	NS
12	1.41±0.09	1.29±0.10	1.11±0.12	1.28±0.16	N vs. JOD = 0.05
14	1.88±0.11	1.64±0.12	1.38±0.14	1.91±0.20	N vs. JOD < 0.01
16	2.21±0.12	1.94±0.13	1.80±0.16	2.18±0.22	N vs. JOD < 0.05
18	2.31±0.12	2.14±0.14	1.99±0.17	2.48±0.22	NS
20	2.38±0.12	2.27±0.13	2.25±0.16	2.56±0.22	NS

Fibroblast cells were scored with each unit taking into account both the outgrowth radius and the cell number. The maximum possible score was 4 U (see Appendix for further details). Abbreviations as in Table IV.
* Mean±SE in arbitrary units.

(Table VIII) but these parameters were not significantly different.

The mean number of cells at the time of confluence (Table IX) was consistently higher in normal cells over the first four deciles of secondary culture but did not reach statistical significance. This parameter has been shown to correlate inversely with age in vivo (28) and in vitro (25, 26, 28). The present observations include counts of the first confluent sheet, which are somewhat obscure because cultures at this specific stage contained both fibroblast and epithelial cells. Although the latter were relatively resistant to detachment by trypsin and tended to have involuted by the time of harvest, they could not, if present in the cell suspension, be discriminated by the electronic cell counter. Beyond this stage, epithelial cells were rapidly overgrown and cell counts reflected cells of fibroblast morphology almost exclusively.

Low density plating efficiency. Plating efficiencies (Table X) showed no significant differences among the four groups at the time of harvest or at four subsequent deciles. However, in all cases there was a consistent downward trend with passage level as has been reported earlier (14, 32).

When five of the best clones that developed after primary plating at 0 MPD were transferred separately to individual 35-mm dishes (Table XI), more normal clones were able to grow to confluence than MOD clones. In clones that were able to attain confluency, the time taken was shorter in normals than in JOD (Table XI). It must be emphasized that these means are biased towards lower values particularly in the three abnormal groups; we could not include the time taken by clones that failed to attain confluence because in these cases this value approached infinity. The number of cells at confluence ranged from 2.2–2.6 × 10^5 with no significant differences between groups. Similarly, when these confluent clones were replated at low density, secondary plating efficiencies in the four groups were 21–26% with no significant differences among them.

The apparently higher total number of cumulative MPD in normal cells before termination of secondary cultures (Table XII) did not reach significance compared to prediabetics, JOD, and MOD. The same held true for the total number of days.

No significant intra- and intergroup differences related to the sex of the donor were found that would influence the results reported here.

TABLE VII
*Primary Culture: Intensity of Outgrowth at Late Stages**

	Normal	Prediabetic	Juvenile-onset diabetes	Maturity-onset diabetes	*P* value
First plateau, *d*	13.24±0.63	15.45±0.69	17.41±0.84	18.21±1.14	N vs. $P < 0.05$; N vs. JOD < 0.01 N vs. MOD < 0.01

Abbreviations as in Table IV.
* Mean±SE.

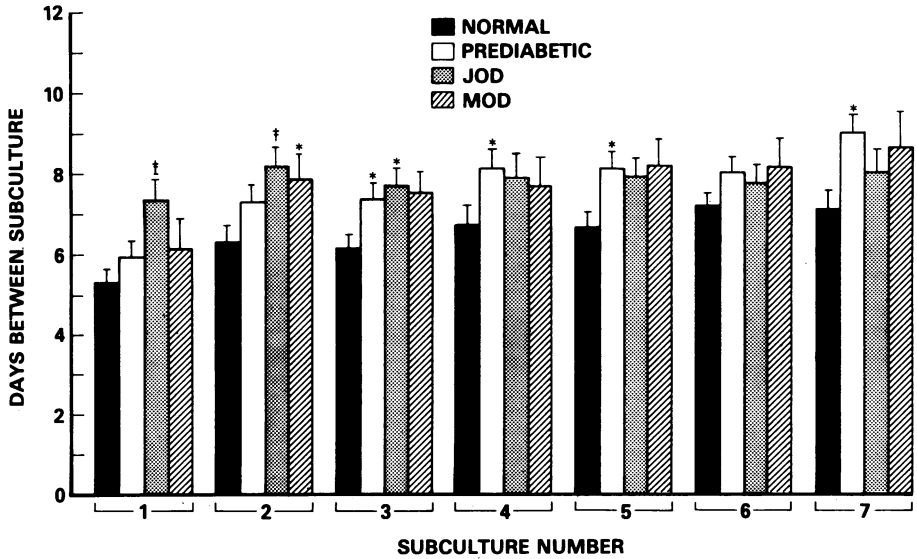

FIGURE 2 Growth vigor of secondary fibroblast cultures measured as the interval required to attain confluence after subculture. A shorter time interval indicates more vigorous growth. Data represent the mean±SEM. Statistically significant differences vs. normal are: $*P < 0.05$ and $\ddagger P < 0.01$.

DISCUSSION

Although the differences were often marginal, these results confirm and extend our initial observations that the growth capacity of skin fibroblasts bearing the diabetic genotype is reduced. A number of growth parameters were examined in primary and secondary cultures, many of which had not been previously assessed. In general, the effects of the diabetic gene(s) are more pronounced in overt diabetics than in those who are presumed to be genetically predisposed but who do not show overt metabolic derangements. Moreover, the detrimental effect of diabetes was evident in both groups with JOD and MOD even though certain decrements seemed to occur in one group more than the other. However, a large degree of variance was encountered, not only in both diabetic groups but also in normals and prediabetics. Studies of several physiologic parameters over the human life-span have demonstrated similar degrees of variance related to the disparity between biological and chronological age (33–35). The large amount of genetic heterogeneity now known to exist in diabetes (1–4) and in every normal individual (36) would also contribute to the variation as well. Because we have averaged various cellular growth parameters across a wide age-span in four groups classified only on the basis of glucose tolerance, the variance is not unexpected.

Intergroup differences in growth capacity occurred during both primary and secondary phases of culture. In the first phase, prior in vivo influences almost certainly persist, whereas in the second, where new

TABLE VIII
*Secondary Culture: Onset of Senescence**

	Normal	Prediabetic	Juvenile-onset diabetes	Maturity-onset diabetes	*P* value
Early					
No. MPD to first refeeding	23.89±2.86	12.57±3.11	12.38±3.81	13.52±5.16	N vs. $P < 0.05$; N vs. JOD < 0.05
No. days to first refeeding	53.15±6.09	30.27±6.61	31.58±8.12	31.37±11.0	N vs. $P < 0.05$; N vs. JOD < 0.05
Late					
No. MPD to slowing	31.32±2.88	26.15±3.13	25.80±3.84	27.68±5.19	NS
No. days to slowing	72.34±6.72	66.14±7.30	67.44±8.96	68.61±12.12	NS

Abbreviations as in Table IV.
* Mean±SE.

TABLE IX
*Secondary Culture: Number of Fibroblasts at Confluence**

MPD level‡	Normal	Prediabetic	Juvenile-onset diabetes	Maturity-onset diabetes	*P* value
			cells per dish × 10^{-6}		
0	1.36±0.12	1.06±0.13	1.10±0.16	1.09±0.22	NS
10	0.72±0.10	0.50±0.10	0.56±0.13	0.62±0.17	NS
19	0.76±0.12	0.50±0.14	0.50±0.17	0.55±0.23	NS
31	0.68±0.15	0.44±0.17	0.35±0.20	0.53±0.28	NS

* Mean±SE cells in 60-mm dish.
‡ 0 MPD represents the harvest of primary cultures and the beginning of secondary culture.

daughter cells have been generated in a relatively uniform environment, the genetic capacity of cells should be more prominently expressed. In any case, several growth parameters were less vigorous in prediabetic and diabetic explants compared to normals for fibroblasts and for epithelial cells at early stages of explantation. It is of interest, therefore, that the emigration rate of cells from explants is reduced as a function of donor age in humans (28, 37) and lower forms (38–40). Curiously, epithelial cells were more vigorous in diabetic and prediabetic explants at late stages before involution. No objective measurement was made of the rate of involution, but in view of the results and currently improved methodology for growing epithelial cells (41), this kind of analysis now deserves further attention.

It may be argued that the differences in secondary cultures ensue as a result of persistent deleterious effects of deranged diabetic metabolism and(or) anatomic pathology in vivo. Two main points argue against this possibility. First, although they showed slower growth at initial explant stages, prediabetic cells and both groups of diabetic cells appeared to catch up at late stages of primary culture so that their cumulative scores and harvest times were equal to those of normals. Second, prediabetic individuals with no overt glucose intolerance, and who were presumably, therefore, free of other severe metabolic derangements, also showed defective fibroblast growth. Moreover, this growth performance was almost always intermediate to that of normals and overt diabetics. These considerations taken together favor the idea that impaired growth in secondary cultures is a consequence of intrinsic genetic mechanisms. On the other hand, the intermediate performance of prediabetic cultures is still in accord with the idea of persistent but more subtle in vivo derangements in prediabetic donors. This raises the possibility that a significant proportion of fibroblast growth reduction in diabetic and prediabetic cultures is in part determined secondarily, that is, as a consequence of long-term hyperglycemic and other metabolic disturbances. In this sense, therefore, diabetes could have a significant "environmental" as opposed to genetic component.

In any case, it is possible that the decreased performance of diabetic and prediabetic cultures is a more direct consequence of two factors, alone or in combination: first, decreased cellularity in the stem cell pool of the dermis which gives rise to the cultured fibroblast; second, a quantitative or qualitative impairment of connective tissue such as glycosaminoglycans or collagen which mechanically restricts emigration of

TABLE X
*Secondary Culture: Plating Efficiency**

MPD level	Normal	Prediabetic	Juvenile-onset diabetes	Maturity-onset diabetes	*P* value
			%		
0	21.99±1.70	18.36±1.85	19.51±2.27	20.02±3.07	NS
10	17.31±0.98	16.98±1.07	16.46±1.31	17.50±1.77	NS
19	17.05±1.36	14.11±1.48	12.80±1.81	16.94±2.45	NS
31	13.41±1.73	10.29±1.92	8.18±2.29	12.79±3.27	NS
40	6.62±1.10	6.38±1.20	5.25±1.48	5.95±2.00	NS

* 250–500 cells were plated into 60- or 100-mm dishes and clones grown and scored as in Methods.

TABLE XI
*Growth of Primary Clones after Subculture**

	Normal	Prediabetic	Juvenile-onset diabetes	Maturity-onset diabetes	*P* value
Percent survivors after picking, %	85.77±5.76	75.61±6.26	78.08±7.68	54.61±10.39	N vs. MOD < 0.05
Recloning time of picked clones, *d*	12.02±0.77	13.55±0.85	14.97±1.04	14.90±1.32	N vs. JOD < 0.05

Abbreviations as in Table IV.
* Mean±SE.

the fibroblast progenitor cell and subsequently limits its ability to give rise to cultured cells. The latter possibility is real (42) but cannot be evaluated on the basis of the present protocol. It clearly warrants further study. The first possibility can be pursued by histological analysis of each skin sample for cellularity. Indeed it has been demonstrated that skin cellularity decreases with chronological age (43, 44) although no data are available for diabetics. However, it will be difficult to identify with certainty and to subsequently quantify the cell or cells that give rise to the cultured fibroblast (45, 46). But even if there were, for example, a twofold reduction in dermal progenitor cells in diabetics, it is hard to imagine that this would lead to slower growth performance over several subsequent stages, especially in secondary cultures.

The reduced colony-forming ability observed in prediabetic cells in an earlier study (14) was not demonstrable in the present work and was not confirmed in a recent series of juvenile diabetic cultures (47). The reasons for this are unknown. It is possible that in selecting the two "best dishes" for harvest we may have been biased against portions of skin with a patchy or clonal growth deficit in prediabetic and diabetic cultures. Indeed such heterogeneity of cellular growth potential has been clearly demonstrated in normal cultures (48). Although a similar procedure was followed in the earlier study (14), fewer dishes were then available to choose from at the time of harvest. But it is more likely that improved growth medium was a factor, as predicted earlier (14). Previously, the mean plating efficiencies on first testing were 12.1 and 7.0% in normals and prediabetics, respectively, whereas in the current study all groups scored about 20%. Thus, improvement in certain nutrient and hormonal factors in the fetal calf serum used in the current work may have corrected deficiencies that were elicited by a nutritionally inferior growth medium in the earlier study. The work of Ham and co-workers (49–51) clearly demonstrates that cellular growth requirements determined under conditions of precisely limited nutrient availability can be masked by supplementation with fetal calf serum in the concentrations used here. Indeed, the idea that in vivo aging may have occurred to a greater extent in subjects with the diabetic genotype so as to compromise the growth capacity of their cells in vitro (12, 14) may just as readily be explained by auxotrophy. Accordingly, diabetic and other poorly replicating cells would have a relative inability to utilize or synthesize growth factors from the medium. Ample precedent exists for this idea in cultured cells derived from individuals with genetic defects, both autosomal recessive and dominant (52). It is also possible that some cells will only reveal a growth deficit when grown in aggregate culture where they attain a precise spatial configuration and(or) interact with other cells (41, 53).

The results in Tables X and XI help to answer the question posed in our previous study (14) whether a universal decrease in growth capacity occurs in diabetic fibroblasts growing out of the explant or whether only a subpopulation is affected adversely. Plating efficiencies were not different between the groups but fewer MOD clones survived after picking. This suggests that a subpopulation of diabetic cells is affected although this only becomes evident after much

TABLE XII
*Secondary Culture: Replicative Life-span of Fibroblast Strains**

	Normal	Prediabetic	Juvenile-onset diabetes	Maturity-onset diabetes	*P* value
Total MPD	52.54±2.24	47.84±2.43	47.12±2.99	46.40±4.04	NS
Total d	180.33±6.72	169.83±7.31	162.48±8.97	156.73±12.13	NS

The total number of days included the 28 d immediately before termination of cultures.
* Mean±SE.

of the replicative lifespan is consumed. These observations support the idea of premature cellular aging in MOD cultures.

The main conclusion of this study is, as before (14), that an effect of the diabetic gene(s) and(or) the resultant metabolic imbalance in vivo is to decrease the growth capacity of some or all fibroblasts either in the initial explant or in cells that grow out subsequently. Similar results have now been found by an increasing number of investigators (54–57). Thus, a diabetic defect can be demonstrated in cells other than the pancreatic beta cell. Although the molecular basis is unknown, it is clear that the basic defect in diabetes need not be restricted to insulin production and(or) release. It is noteworthy that a diversity of defects has recently been demonstrated in cultured fibroblasts derived from subjects with premature aging syndromes which also feature overt diabetes and(or) insulin resistance (12, 13, 25, 54, 58). Indeed, these studies clearly indicate that diabetes can be expressed in peripheral cells in association with or consequent to premature cellular senescence. It remains to be proven whether the present results relate to cellular senescence, auxotrophy, or whether these two phenomena are somehow interdependent.

In conclusion, a persistent, heritable abnormality is present in cells originating from mesenchymal tissue of an extrapancreatic site of donors with overt diabetes or the predisposition to this disease. This system should prove useful in exploring the clinical and genetic heterogeneity of diabetes, the mechanisms which lead to hyperglycemia and other metabolic derangements, and also the propensity that affected individuals have to earlier onset and more severe development of age-dependent degenerative diseases.

APPENDIX

Study protocol and glossary of terms (see Fig. 1)

Primary culture. The interval from initial explantation of skin fragments until harvest of cells, i.e. first subculture.

EXPLANTATION

The time at which explants were placed into medium and incubation at 37°C began (time zero).

FIBROBLAST CELLS

Elongated, spindle-shaped cells with frequent angular processes that tended to emigrate in uneven growth patterns from the circumference of fragments.

SCORING OF INITIAL DAY OF OUTGROWTH AND CUMULATIVE PERCENT OUTGROWTH IN FRAGMENTS

Cultures were examined with an inverted microscope (×40). The first visible outgrowth of fibroblast-like cells was noted in each fragment. Then the number of fragments showing any such growth over the period of observation was scored as a percent of total number of fragments.

Maximum rise. The maximum percent of fragments showing fibroblast growth.

First day of plateau. The earliest time in days at which the maximum percentage of fragments showed growth.

Cumulative intensity of growth per fragment. The microscopic field was calibrated and had a diameter of 5.12 mm. Each skin fragment was positioned in the center of the field and scored on a scale of 1–4 by specific objective criteria, taking into account both the distance of cell emigration from fragments and the number of cells: (1) 1–100 cells at a radius of 0.32 mm from edge of fragment; (2) >100 cells grown to 0.64–0.96 mm from edge of fragment; (3) Cells almost confluent to a radius of 0.96 mm from fragment; (4) Cells confluent and approaching edge of Petri dish. The maximum possible score for fibroblast outgrowth is 4.

Harvest day. The day at which cellular sheets in two dishes of explants became confluent.

Epithelial cells. Epithelial cells were distinguished from fibroblasts by their round to polygonal shape and higher refractility under light microscopy. In contradistinction to fibroblasts, epithelial cells tended to emigrate in dense confluent sheets around the entire circumference of each fragment. They appeared earlier than fibroblasts but generally ceased dividing about 10–14 d and began to involute after 14 to 21 d, usually before the time of harvest. At this time they were much more resistant to trypsin than fibroblast cells and were detached only minimally by the enzymatic procedure. Epithelial cells were identified and scored separately during the primary culture period on a scale of 1–10, where each unit represented an outgrowth radius of 0.24 mm. Scoring was done only to day 14 because of cell involution. Maximum possible score per fragment is 10. Percent outgrowth in fragments, maximum rise, and earliest day of plateau determined as for fibroblasts.

Secondary culture. The beginning of continuous subcultivation after initial harvest of cells arising from explants.

MPD. The average number of times that the cell population doubles without reference to the true number of cell generations that may have occurred. That is, some cells may not divide at all, whereas others may divide several times during 1 MPD.

First subculture. The transfer of initial confluent sheets of cells from primary explant dishes to secondary culture (count 2 MPD).

Second subculture. Cells subcultured at a 1:4 dilution with respect to the surface area of dishes (count 2 MPD → cumulative total of 4 MPD). Cells were banked in liquid nitrogen storage at this stage.

Third subculture to termination. From this stage onward, cells were subcultured each time they became confluent at a 1:8 dilution. (Count 3 MPD with each such manipulation→ cumulative totals of 7, 10, 13, and so forth.)

Counting of interval of subculture. The days between subcultures as a measure of growth vigor, i.e., shorter time interval indicates more vigorous growth.

Time of first refeeding (in calendar days and MPD). When cells first failed to become confluent in 7 d and hence required fresh medium; this is an early sign of senescence.

First slowing of growth (in calendar days and MPD). When cells that required refeeding slowed even further and needed significantly >7 d and one medium change to attain confluency; this is a later sign of senescence.

Plating efficiency after low density plating. Measure of ability of an individual cell to attach and form a visible colony within 2 wk.

Recloning time. The interval in days after picking of pri-

mary clones (plated at 0 MPD only), and transfer into 35-mm dishes for cells to form a confluent sheet.

Secondary plating. The replating of primary clones that were able to survive picking and grow to confluence. After 2 wk of growth, colonies were stained and scored to determine the secondary plating efficiency.

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