Abnormalities in Proliferation and Protein Synthesis in Skin Fibroblast Cultures from Patients with Diabetes Mellitus

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SUMMARY

Several aspects of in-vitro cell growth and protein synthesis were assessed in cultures of skin fibroblasts from subjects with juvenile-onset diabetes mellitus (JODM) or adult-onset diabetes mellitus (AODM) and from age-matched nondiabetic controls (C). There was an inverse correlation between increasing age and both the log-phase doubling rate and saturation density at confluence in C fibroblasts. JODM and AODM cells had a reduction in both indices of cell population growth in comparison with age-matched C fibroblasts. Fibroblasts grown in the presence of 0.3 μM hydrocortisone were stimulated to grow more rapidly and to a greater saturation density. Stimulation of cell division by hydrocortisone accentuated the abnormalities in growth of JODM and AODM fibroblasts.

Total protein and collagen synthesis was measured when the fibroblasts had grown to confluency in medium with or without hydrocortisone. Hydrocortisone did not produce a significant change in total protein and collagen synthesis per cell by C fibroblasts. Fibroblasts from AODM had a 180 per cent increase in total protein and collagen synthesis in the presence of hydrocortisone. In contrast, total protein and collagen synthesis decreased 40 per cent in fibroblasts from JODM when grown in the hydrocortisone medium. These studies indicate that skin fibroblast cultures from patients with diabetes exhibit abnormalities in cell proliferation. Furthermore, hydrocortisone appears to unmask differences in protein synthesis that distinguish JODM and AODM fibroblasts in culture. DIABETES *26*:284-90, April, 1977.

Diabetes mellitus is a disease characterized by insulin deficiency, hyperglycemia, and premature degenerative changes in large blood vessels, capillaries, and peripheral nerves. There are conflicting clinical and experimental data on the interrelationship between metabolic disturbances and tissue complications of the diabetic state. Martin,[1] Vracko,[2] and Goldstein[3,4] examined skin fibroblasts from diabetics in tissue culture and found that their in-vitro replicative life span was reduced when compared with age-matched control cells. Since decreased total cell population doublings are characteristic of cultured fibroblasts from old donors[1,5] and also of fibroblasts as they age in vitro,[6,7] the diabetic state resembles a form of premature aging.

The studies reported here were initiated to quantitate abnormalities in cell growth and protein synthesis in fibroblasts from diabetics. Collagen was measured as a marker protein of the synthetic capability of fibroblasts. Since many of the steps of collagen synthesis are well characterized,[8] abnormalities in collagen production should allow more definitive testing of cellular abnormalities in fibroblasts from diabetics.

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Accepted for publication October 14, 1976.

MATERIALS AND METHODS

Materials. The following items were employed:

Eagle's minimal essential medium (MEM) and nonessential amino acid mixture (Microbiological Associates), penicillin-streptomycin solution and fetal calf serum (Grand Island Biological Company), ascorbic acid (Cal Biochem), collagenase, ABC form III (Advanced Biofactures), dimethyl sulfoxide, hydrocortisone and diamino benzoic acid (Sigma), trypsin (Worthington), [2,3-^{3}H]-L-proline, specific activity 30 Ci./mmole, and [2-^{3}H]-glycine, specific activity 5 Ci./mmole (New England Nuclear).

Methods. After informed consent was obtained, the skin of the deltoid region was infiltrated with 1 per cent xylocaine and 6-mm. punch biopsies were taken. The specimens were minced with scissors into 10-20 small pieces and allowed to adhere to the surface of a 60-mm. plastic tissue-culture dish (Falcon Plastics). The cells were grown in Eagle's MEM supplemented with nonessential amino acids,[9] 10 per cent (v/v) fetal calf serum, 100 μU./ml. penicillin, and 100 μg./ml. streptomycin at 37° C. in an atmosphere of 95 per cent air and 5 per cent CO_2. Fibroblasts from these explants were subcultivated, harvested, and frozen, usually at the fourth or fifth subcultivation or passage, in liquid nitrogen in Eagle's MEM containing 10 per cent (v/v) fetal calf serum and 10 per cent (v/v) dimethyl sulfoxide. All studies were performed on cells that had been stored in liquid nitrogen and had not undergone more than 15 in-vitro population divisions.

Fibroblasts were retrieved from freeze storage, rapidly thawed, and maintained in 16-ounce glass prescription bottles in Eagle's MEM supplemented with 1 gm./L. glucose, nonessential amino acids,[9] 100 μU./ml. penicillin, 100 μg./ml. streptomycin, and 10 per cent (v/v) fetal calf serum. At the start of an experiment, 2-4$\times 10^6$ cells were removed from the culture vessel by brief incubation with 0.25 per cent trypsin solution (in phosphate-buffered saline, pH 7.4), centrifuged at 150 g x 10 minutes, and resuspended in culture medium. Approximately 75,000 cells in 3 ml. of medium were dispensed into each 60-mm. culture dish (Falcon Plastics). The replicate cultures were incubated overnight. The following morning the medium was changed in half the dishes to one containing 0.3 μM hydrocortisone. Hydrocortisone was dissolved in ethanol before addition to the culture medium. In the remaining control cultures, medium contained 0.05 per cent (v/v) ethanol. On days 2, 4, 6, 9, and 12, cultures were removed, washed with 0.15 N NaCl, and air-dried for subsequent DNA determinations. Fresh medium was added to the remaining culture dishes.

The DNA content was determined after scraping the dried cell layer three times in 1 ml. of cold 5 per cent trichloroacetic acid (TCA). The insoluble residue was collected by centrifugation, washed once with 0.1 M potassium acetate in absolute ethanol, and air-dried. The residue was analyzed in toto for DNA content with recrystallized diamino benzoic acid in a fluorometric method.[10]

Medium was removed on day 12 and replaced with Eagle's MEM not supplemented with nonessential amino acids but containing 10 per cent (v/v) fetal calf serum, 5.0 μg./ml. ascorbic acid, and 5.0 μCi./ml. of ^{3}H-proline and ^{3}H-glycine. After a 24-hour incubation period, medium was removed and dialyzed against four changes of 0.1 per cent acetic acid.

Protein synthesis by the fibroblasts was assessed by the accumulation of radioactive protein in the culture medium. This compartment represents about 20 per cent of the total protein synthesized by fibroblasts; approximately 40 per cent of the total medium protein is collagen. Thus, the measurements do not reflect total synthesis by fibroblasts but actually the synthesis of secreted proteins. However, in the many different cell strains that we have examined, the medium compartment parallels total cellular synthesis. In practice, the radioactivity in aliquots of the dialyzed medium was measured by scintillation spectroscopy to determine total protein synthesis. Radioactive collagen of the medium compartment was determined by the collagenase technique of Peterkofsky.[11] All samples were dissolved in Aquasol (New England Nuclear), and a correction for quenching in TCA and dilute acetic acid was entered into the final calculation. A radioactive purified collagen standard was run with each collagenase digestion to normalize minor variations in the percentage of collagen digestion.

Patient selection. Fibroblast cultures were derived from diabetic patients without clinically significant renal or peripheral vascular disease. At the time of the biopsy, all patients were receiving insulin and had varying degrees of control of their diabetes. Juvenile-onset diabetes mellitus (JODM) was defined as ketoacidosis-prone hyperglycemia with an acute onset of symptoms during adolescence or early adulthood. Their disease had been active for between 7 and 20 years and required between 40 to 80 U. of insulin. Adult-onset diabetes mellitus (AODM) was characterized by a gradual onset of symptoms after age 30 and was not ketoacidosis-prone. These subjects had

been taking insulin for no longer than five years but probably had hyperglycemia for 10-15 years. Control fibroblast cultures (C) were derived from patients in the Endocrine-Metabolic Clinic who were without fasting hyperglycemia (plasma glucose < 100 mg./100 ml.) and with no family history of diabetes

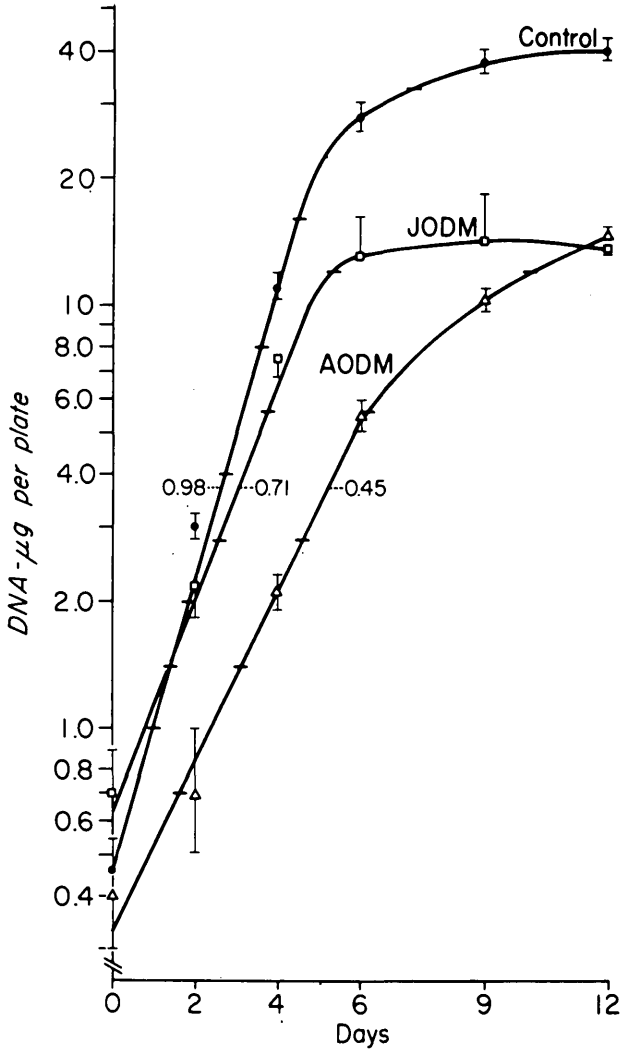

FIG. 1. Growth curves of representative C (24-year-old female with a thyroid nodule), JODM (23-year-old female with diabetes of seven years' duration), and AODM (a 60-year-old man with obesity and diabetes for 10 years) fibroblast strains. Results are expressed as the mean ± 1 S.D. (vertical lines) of three determinations. Growth rate was calculated by curve-fitting the equation: $\ln y = \ln a + bx$, where y is the DNA and x are the days of growth (0, 2, 4, 6). The cell population doubling rate was obtained by: b/ln 2; representative values are numbered by each growth curve. The horizontal lines drawn through each growth curve represent one population doubling. Note that the control strain underwent five population doublings during log-phase growth, while the diabetic lines had four doublings.

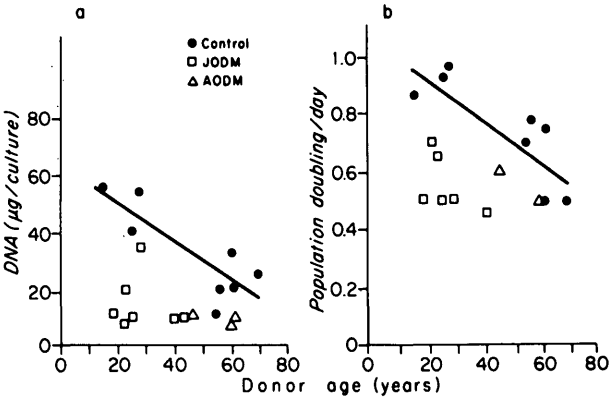

FIG. 2. Cell density at confluency (a) and cell doubling rate (b) of JODM, AODM, and C fibroblasts plotted as a function of donor age. (a) $r = -0.83$, $p < 0.01$, (b) $r = -0.84$, $p < 0.005$.

among first-degree relatives. No attempt was made to exclude control patients that had a family history of diabetes beyond first-degree relatives.

RESULTS

Growth curve. Representative growth curves of control, JODM, and AODM fibroblasts in control medium are shown in figure 1. The log-phase growth rate was the linear portion of the growth curve and lasted approximately six days in all strains tested. Log-phase growth ended when cell-to-cell contact occurred in the culture dish. Further growth, designated postconfluent growth, occurred at a slower rate than log-phase growth. The DNA determination at day 12 was defined as the postconfluent cell density. There were approximately five population doublings during log-phase growth in the control cell strain while four doublings occurred in the diabetic strains. There was no more than one population doubling in the postconfluent growth in fibroblasts from control or diabetic subjects.

Fibroblast growth characteristics. Since the cumulative number of cell doublings in vitro has been shown to be affected by both in-vivo and in-vitro aging,[1,5-7] our data are expressed relative to the donor's age. With increasing donor age, control fibroblasts had a reduction in population doubling rate during the exponential phase of growth ($r = -0.83$) and in the cell density at confluence ($r = -0.84$) (figure 2).

JODM fibroblasts had a 40 per cent reduction in doubling rate ($p < 0.005$) and 70 per cent reduction in cell density at confluence ($p < 0.005$) when compared with age-matched controls (figure 2, table 1). The length of time JODM cells remained in log-phase

TABLE 1

Growth characteristics of JODM, AODM, and age-matched control fibroblasts in medium with (HC) and without (C) hydrocortisone supplementation*

Group	N	Age (years)	Growth rate Cell doublings per day C	Growth rate Cell doublings per day HC	Cell density at confluence μg. DNA C	Cell density at confluence μg. DNA HC
JODM	7	29 ± 10	0.56 ± 0.11	0.66 ± 0.12	15.9 ± 10	23.5 ± 11
P		NS	< 0.005	< 0.005	< 0.005	< 0.005
Controls	3	23 ± 6	0.93 ± 0.05	1.01 ± 0.12	50.4 ± 7.5	68.2 ± 11
AODM	3	55 ± 10	0.57 ± 0.12	0.66 ± 0.12	11.7 ± 3.7	13.6 ± 4.3
P		NS	NS	NS	< 0.05	< 0.005
Controls	5	64 ± 6	0.65 ± 0.14	0.77 ± 0.16	23.8 ± 8.4	39.1 ± 11

*Data expressed as mean ± 1 S.D. Fibroblasts were incubated throughout the 12-day growth period in medium containing 0.3 μM hydrocortisone (HC) or 0.05 per cent ethanol (C).

growth was similar to that of control cells. AODM cells did not show a significant reduction in the growth rate, but the cell density at confluence was reduced by 50 per cent ($p < 0.05$). AODM cells never showed a clear demarcation between the log and confluent phases (figure 1).

Protein synthesis. Amino acid incorporation into medium proteins was linear during the 24-hour labeling period (data not shown). In control cells, including fibroblasts from fetal donors, both collagen synthesis (8.7 ± 5.3 cpm/μg. DNA) and total protein synthesis (22.5 ± 7.6 cpm/μg. DNA) remained constant independently of donor age (figure 3). Therefore the diabetic strains were compared to control values independent of donor age.

JODM fibroblasts synthesized significantly more collagen ($p < 0.01$) and total protein ($p < 0.005$) per microgram DNA than did control cell strains (table 2). AODM cells showed a slight increase in protein synthesis per microgram DNA ($p < 0.05$) but did not differ from control cultures in the amount of collagen synthesized per cell.

Effect of hydrocortisone. Hydrocortisone has been reported to stimulate cell growth in a number of fibroblast systems.[12-14] As will be reported subsequently,[15] in the presence of 10 per cent fetal calf serum, 0.3 μM hydrocortisone produced maximal growth stimulation in human diploid fibroblasts from nonfetal donors. In the presence of hydrocortisone, control fibroblasts increased log-phase growth rate 10-20 per cent and cell density at confluence 40-80 per cent greater than cells grown in the absence of hydrocortisone (figure 4a, table 1). A similar percentage increase in growth parameters occurred in JODM fibroblasts. For both JODM and control cultures, log-phase growth persisted for six days in the presence

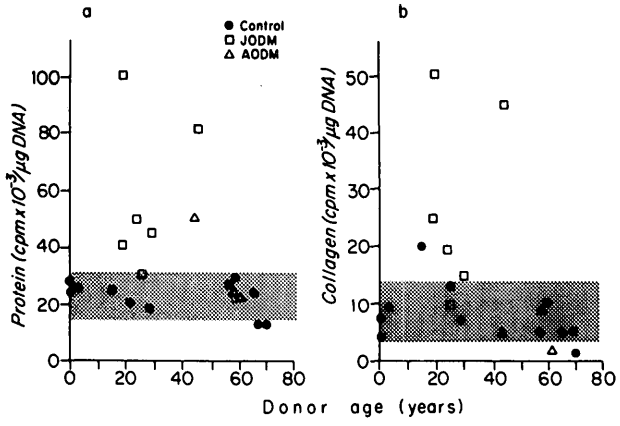

FIG. 3. Total protein (a) and collagen synthesis (b) in the medium compartment expressed per microgram DNA by JODM, AODM, and C fibroblasts plotted as a function of donor age. The shaded area is the mean ± 1 S.D. for C fibroblasts. (a) x = 22.5 ± 7.6; (b) x = 8.7 ± 5.3. The cell strains from fetal donors are included to illustrate that neither total protein nor collagen synthesis per cell varied with changes in the donor's age.

TABLE 2

Total protein and collagen synthesis per cell in JODM, AODM, and control fibroblasts in medium with (HC) and without (C) hydrocortisone supplementation*

Group	N	Total Protein cpm × 10^{-3} /μg. DNA C	Total Protein cpm × 10^{-3} /μg. DNA HC	Collagen cpm × 10^{-3} /μg. DNA C	Collagen cpm × 10^{-3} /μg. DNA HC
JODM	6	58.9 ± 27.5	49.6 ± 22.9	27.8 ± 16.9	18.7 ± 13.9
p		<0.005	<.01	<0.01	<0.05
Control	8	21.2 ± 8.1	25.2 ± 9.7	7.8 ± 4.5	9.6 ± 6.1
p		< 0.05	<0.005	NS	< 0.01
AODM	3	36.4 ± 13.2	98.4 ± 49.9	6.6 ± 3.2	17.3 ± 6.7

*Data expressed as mean ± 1 S.D. Fibroblasts were incubated throughout the growth period in the presence of hydrocortisone (HC) or 0.05 per cent ethanol (C). The incubation with ^{3}H-proline and ^{3}H-glycine on day 12 was performed in medium containing 5 μg./ml. ascorbic acid plus ethanol or hydrocortisone.

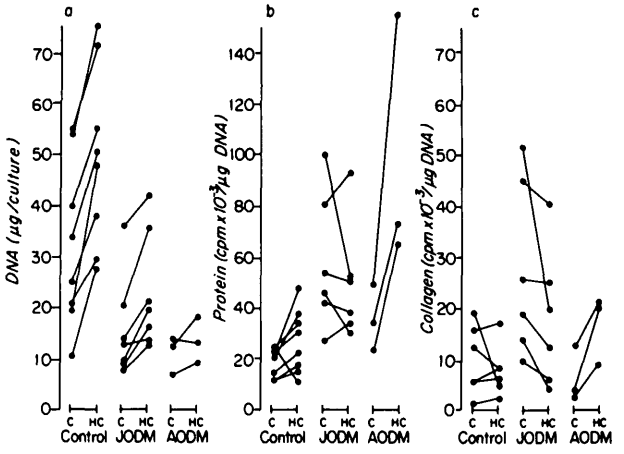

FIG. 4. The effect of medium containing hydrocortisone (HC) or ethanol (C) on cell density at confluency (a), total protein synthesis (b), and collagen synthesis (c) per microgram DNA in JODM, AODM, and C fibroblast cultures.

of hydrocortisone. The response of AODM cells was more variable and the increments in doubling rate and cell density at confluence were less than for age-matched control cells.

In the presence of hydrocortisone, control cells synthesized about 25 per cent more total protein and 6 per cent more collagen per microgram DNA (figures 4b and 4c, tables 2 and 3). The rate of protein synthesis in control cells remained linear for the 24-hour labeling period when hydrocortisone was present (data not shown). JODM cells responded to hydrocortisone with a 16 per cent decrease in total protein synthesis and a 36 per cent decrease in collagen synthesis. In contrast, AODM cells showed a 160 and 180 per cent increase in total protein and collagen synthesis, respectively.

DISCUSSION

Although many of the acute metabolic abnormalities in diabetes mellitus are the consequence of insulin deficiency, the mechanisms underlying the connective tissue changes of the diabetic state are poorly understood. The in-vitro studies[1-4] of plating efficiency and replicative life span of fibroblast cultures suggest that the diabetic state is associated with cellular characteristics of aging. Furthermore, measurements of capillary basement-membrane thickness in nondiabetic subjects show a direct correlation with increasing age of the donor;[16] thus, the increased membrane thickness found in diabetics may also reflect an accelerated aging process. However, these studies do not indicate whether the aging characteristics found in diabetic fibroblasts are an expression of a genetic abnormality in the fibroblast or the consequence of a prolonged exposure to an abnormal metabolic environment. The report by Goldstein[3] that fibroblasts from genetic prediabetics have a reduction in plating efficiency supports the genetic-abnormality hypothesis.

Recent genetic and clinical studies indicate that AODM and JODM are distinct genetic diseases.[17] Tattersall has suggested that certain forms of AODM are inherited in an autosomal-dominant manner.[18] JODM occurs sporadically and often without a family history of the disease. Studies of identical twins[19] suggest that environmental, autoimmune, or viral factors[20,21] may play a role in the etiology of JODM. Thus, all forms of diabetes should not be grouped as one disease entity stemming from a common inherited abnormality. The results in this present study support this concept.

In our experiments, specific abnormalities in cell proliferation and protein synthesis have been sought that, once characterized, could be studied at a more fundamental level. It was found that both JODM and AODM have abnormal cell population growth characteristics. The rate of growth during the exponential phase was reduced in JODM, while the final cell density was diminished in both JODM and AODM. Since both of these parameters also decrease in normal fibroblasts with increasing donor age, diabetic cells appear to be similar to cells cultured from aged donors.

Further studies of enzyme heat-denaturation curves,[22] DNA repair rates,[23] and HL-A expression[24] will be required to determine if other characteristics of aged cells are present. Nevertheless, certain characteristics were found to differ from aged control cells. For example, both the total protein and collagen synthetic capacity of nondiabetic fibroblasts did not change with age; however, both were greater in JODM than in nondiabetic cells, thus differentiating

TABLE 3

Effect of hydrocortisone on total protein and collagen synthesis in JODM, AODM, and control fibroblasts*

Group	N	Total protein	Collagen
JODM	6	0.84 ± 0.24	0.64 ± 0.27
p		< 0.025	< 0.05
Control	8	1.25 ± 4.2	1.06 ± 0.46
p		< 0.005	< 0.005
AODM	3	2.60 ± 0.57	2.80 ± 0.45

*Data expressed as: cpm × $10^{-3}/\mu g$. DNA in hydrocortisone medium ÷ cpm × $10^{-3}/\mu g$. DNA in control medium, mean ± 1 S.D.

JODM cells from aged nondiabetic cells.

Furthermore, in certain respects JODM and AODM cells differed from each other. Whereas some of the growth characteristics were similar in JODM and AODM cells, the response to hydrocortisone differed. Although hydrocortisone caused an inhibition of protein synthesis in JODM, a striking stimulation of synthesis was found in AODM cultures.

The mechanism for the altered rates of protein synthesis in diabetic fibroblasts in the basal and hydrocortisone-treated state is unclear. In fact these studies indicate only that the accumulation of radioactive protein is altered in the diabetic fibroblast. The accumulation rate is affected by the amino-acid pool size, endogenous rates of protein catabolism, and inherent translational efficiency of the cells' protein synthetic machinery (true synthetic rate). Any of these parameters may be at fault within the diabetic fibroblast. For example, senescent WI-38 fibroblasts at confluence have a high rate of protein degradation,[25] which might alter the rate of protein accumulation or the specific activity of the isotope used for labeling.[26] The defect might be at the translational level, since abnormalities in ribosomal subunit association have been demonstrated in the muscle of diabetic animals.[27] Macieira-Coelho[28] has demonstrated that hydrocortisone stimulates the synthesis of polyribosomes in normal fibroblasts. Thus, another explanation is that a cellular abnormality in polyribosomal synthesis or function exists in diabetic fibroblasts. In the case of JODM, the consequence of this abnormality would become exaggerated when cell division is stimulated by hydrocortisone because the demand on ribosomal activity is maximal at this time. The resultant effect would be a reduction in the synthesis of "luxury proteins," such as collagen, in favor of required cellular proteins.

The value of identifying abnormalities in protein accumulation in diabetic fibroblasts is the experimental feasibility of studying amino-acid pool size, protein degradation, and translational efficiency in the cells. It should also be possible to determine if the abnormalities found in the diabetic fibroblasts are present prior to the onset of glucose intolerance or if they are related to the length or severity of the diabetic state. At this point, however, these early studies support the clinical evidence that diabetes mellitus, in many respects, resembles premature aging, and furthermore, that AODM and JODM are distinct disorders.

ACKNOWLEDGMENTS

This investigation was supported in part by USPHS grants AM 02456, AM 05020, and AM 15312, and by the Kroc Foundation, UW 63-2986. Dr. Rowe is a fellow of the Helen Hay Whitney Foundation. Dr. Fujimoto is a recipient of Research Career Development Award AM 47142 from NIAMDD.

The excellent tissue culture assistance of Jeanette Teague and the technical assistance of Amy Wong are gratefully acknowledged.

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