Effects of Glucose and Sorbitol on Proliferation of Cultured Human Skin Fibroblasts and Arterial Smooth-muscle Cells

John L. Turner, M.D., and Edwin L. Bierman, M.D., Seattle*

SUMMARY

To determine effects of metabolic abnormalities associated with diabetes mellitus on proliferation of diploid human cells, cultured human skin fibroblasts and arterial smooth-muscle cells were grown in media containing added glucose in the range often seen in diabetic subjects (10 to 30 mM, 180 to 550 mg./dl.). "High" glucose media enhanced proliferation of fibroblasts, with an "optimal" response at about 18 mM (325 mg./dl.). Equimolar sorbitol gave similar results, with the greatest increase in proliferation occurring at about the same concentration as for glucose (19 mM). Since neither equimolar mannitol nor sucrose produced such effects consistently, these results cannot be explained solely on the basis of hyperosmolarity. In contrast, arterial smooth-muscle cells failed to show a consistent growth response in the presence of either added glucose or sorbitol. These results suggest that studies with cultured human cells may be useful in assessment of responses to components of the disordered metabolic milieu of diabetes. Such studies of arterial smooth-muscle cells should also be useful for investigation of the mechanism of atherosclerosis in diabetes. DIABETES *27*:583-88, May, 1978.

INTRODUCTION

Controversy still surrounds the issue of whether or not hyperglycemia contributes to the development of the long-term vascular concomitants of diabetes mellitus in man.[1-3] It has been suggested that the chronic manifestations of the diabetic syndrome may be related to an inherent cellular abnormality that results in "premature aging."[4] This has been interpreted by some to suggest that the hyperglycemia (or other metabolic derangements of diabetes) may have less to do with the chronic manifestations than the genetic predestination of a particular patient. Indeed, a good correlation between the degree of hyperglycemia and the incidence or progression of vascular disease seems lacking.[2,3] Still, there is some evidence that certain of the long-term concomitants may be influenced by the severity of hyperglycemia,[5,6] and many clinicians believe that meticulous control of the blood glucose level will be helpful in delaying or preventing vascular disease.

The development of atherosclerosis, associated with the majority of deaths in diabetes,[7] is believed to result in part from proliferation of arterial smooth-muscle cells during early phases of plaque formation.[8,9] In addition, both large-vessel disease and microvascular disease have been associated with elevated blood glucose concentrations.[1,10] Use of cell culture techniques affords an opportunity to study directly proliferative and metabolic responses of homogeneous populations of diploid human cells grown in controlled nutritional environments, circumventing some of the limitations of systems requiring intact organisms or whole tissues with mixtures of cell types. By use of this technique, studies of effects of the anbormal metabolic milieu associated with the diabetic state have been initiated.

However, since there is a relative paucity of information on precise nutritional requirements of cultured human diploid cells, preliminary studies were per-

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From the Division of Metabolism, Endocrinology, and Gerontology, Department of Medicine, University of Washington, Seattle, Washington.

Address reprint requests to Dr. Edwin L. Bierman, Division of Metabolism, Endocrinology, and Gerontology, Department of Medicine RG-20, University of Washington, Seattle, Washington 98195.

*Dr. Turner's current address: Endocrine Section, Department of Medicine, 522 Johnson Pavilion G2, University of Pennsylvania, Philadelphia, Pennsylvania 19104.

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formed to establish baseline conditions. Most of the "minimal essential" media used in tissue culture have been derived for established cell lines and have been designed to support, and not necessarily to promote, cell growth;[11] indeed, there has recently been renewed interest in nutritional aspects of cultured cells.[12]

Therefore, in the present study, the effects of medium glucose concentrations similar to those often found in diabetic subjects on proliferation of cultured human skin fibroblasts were tested, since there is recent evidence that certain "small nutrient" molecules may be important in the control of cell growth and proliferation.[13,14] In addition, the effect of glucose on proliferation of cultured human arterial smooth-muscle cells was tested as well, both because of the importance of this cell in atherogenesis[8,9] and because it has been shown that various metabolic derangements occur in arterial tissues as a consequence of high medium glucose concentrations.[15,16]

MATERIALS AND METHODS

Human arterial smooth-muscle cell cultures were established from explants of arterial specimens from two sources. Segments of thoracic aorta were obtained from children undergoing surgery for coarctations of the aorta. Segments of lower-extremity arteries were obtained from older patients during reconstructive arterial surgery performed for occlusive arterial disease. The older patients included persons both with and without known diabetes as determined by standard clinical criteria. Cultures were established according to the method of Ross[17] as previously described from this laboratory.[18-20] Human skin fibroblast cultures were established similarly, with specimens obtained from small punch biopsies of skin of the anterior thigh of normal and juvenile-onset-diabetes donors. Explants were grown in Falcon tissue culture flasks (250 ml.) containing enough modified Dulbecco-Vogt medium[18] supplemented with either 10 per cent heat-inactivated fetal calf serum (for fibroblasts) or 10 per cent heat-inactivated pooled human serum (for smooth-muscle cells) to just cover the explants. The flasks were incubated at 37° C. in a humid atmosphere of 95 per cent air and 5 per cent carbon dioxide. Small amounts of medium were added every four to five days until the cells became confluent. At that point, the medium was removed and the cells were washed with 5 ml. of a Versene buffer solution, gently trypsinized with 5 ml. of a solution of trypsin in Versene buffer, and incubated for 10 minutes at 37° C. Cells were then seeded into new flasks in 10 ml. of incubation medium. Half the medium was removed and replaced with fresh medium twice a week.

For the proliferation experiments, cells were used after two to 10 passages. Following trypsinization, cells were seeded in 2 ml. medium in small Falcon plastic tissue culture dishes (35 mm.) with 1 to 5 × 10^4 cells used for fibroblasts or 10 to 25 × 10^4 cells for smooth-muscle cells. During a two-day basal period (day 0 to day 2), fibroblasts were grown in modified Dulbecco-Vogt medium (5 mM glucose) containing only 1 per cent fetal calf serum so that little, if any, growth occurred. The more fastidious human smooth-muscle cells required 10 per cent pooled human serum to remain viable under the same conditions. At day 2, experimental media were substituted. These contained 5 per cent fetal calf serum for fibroblasts or 10 per cent pooled human serum for smooth-muscle cells. The 5 per cent fetal calf serum concentration was found in preliminary experiments to reliably support growth of the fibroblast strains and was chosen to be low enough so as not to mask a potential growth-promoting effect of the added glucose. Similarly low concentrations of serum could not be used for the arterial smooth-muscle-cell experiments, since some strains did not grow or even died in the presence of 5 per cent serum; thus, all arterial smooth-muscle-cell experiments were performed in media containing 10 per cent pooled human serum. Glucose was added to the 5 mM present in the basal medium to give concentrations in most experiments of 10 mM, 20 mM, and 30 mM, chosen to be representative of glucose values in a range (90 to 540 mg./dl.) often seen in plasma of diabetic subjects. To control for a possible effect of hyperosmolarity of the experimental media on proliferation, equimolar sorbitol was used in some experiments. To maintain glucose concentrations of the medium constant in the presence of cell glucose utilization, medium was changed daily. Observations in this laboratory suggest that glucose consumption by cultured cells can be substantial if cell numbers are large and medium is not changed for two to three days.[21] Medium glucose was measured with a Beckman glucose analyzer.

Counts of numbers of trypsinized cells were made with a Fuchs-Rosenthal counting chamber at intervals during the growth period until stationary phase was reached (usually by days 10 to 12 for fibroblasts and days 12 to 14 for arterial smooth-muscle cells). Two counts each of duplicate dishes were made for each glucose or sorbitol concentration on each counting day.

Data were analyzed by comparison of changes in cell numbers seen in the various experimental conditions to the increment in cells seen in the basal medium. This comparison could then be expressed as a per cent change in cell number to a particular point in time. Thus, since an internal control (i.e., cells of the same strain grown in basal medium in the same experiment) was utilized in every experiment, the data points from several experiments could be directly compared. Results were then analyzed statistically using the paired *t* test.

RESULTS

Preliminary results suggested a stepwise incremental effect of increasing medium glucose concentrations on proliferation of fibroblasts up to about 20 mM. Above that glucose concentration, further enhancement of proliferation usually failed to occur, or, in some experiments, there was even a decremental effect, as illustrated in table 1. Subsequent experiments (n = 17) confirmed that greater cell numbers resulted when cells were grown in media containing "high" glucose concentrations (figure 1). In every case but one, cell numbers were greater in "high glucose" (18.4 ± 6.5 mM, 331 ± 117 mg./dl.), with a mean increase of (+) 30 per cent ($p < 0.001$). These 17 strains included cells from three diabetic subjects and 14 nondiabetics; no difference in growth behavior of the diabetic strains was apparent.

TABLE 1

Effect of glucose on cell proliferation

Glucose			10^4 Cells/dish					
mM	mg./dl.	Day	0	1	2	3	6	12
5	90		1.00	0.63	0.78	0.63	3.38	8.95
10	180		—	—	—	1.22	4.05	12.95*
20	360		—	—	—	0.97	3.39	15.70†‡
26	470		—	—	—	0.78	3.27	14.40†

*$p < 0.05$ vs. 5 mM glucose.
†$p < 0.001$ vs. 5 mM glucose.
‡$p < 0.05$ vs. 10 mM glucose.

Data from one representative experiment illustrating the effect of various medium glucose concentrations on proliferation of cultured skin fibroblasts. Cell counts are Means of duplicate counts from replicate dishes. Statistical analysis performed by the *t* test for two means.

Experiments using fibroblasts grown in media containing added sorbitol gave results similar to those with added glucose (figure 2). The mean increase in cell number seen in 10 strains in 13 experiments was (+) 42 per cent ($p < 0.005$). The average sorbitol concentration that produced a maximum enhancement of proliferation was 19 ± 7 mM, a value virtually identical to that found with glucose. Similarly, the modest inhibitory effect sometimes seen with higher concentrations of glucose was often observed with sorbitol, too.

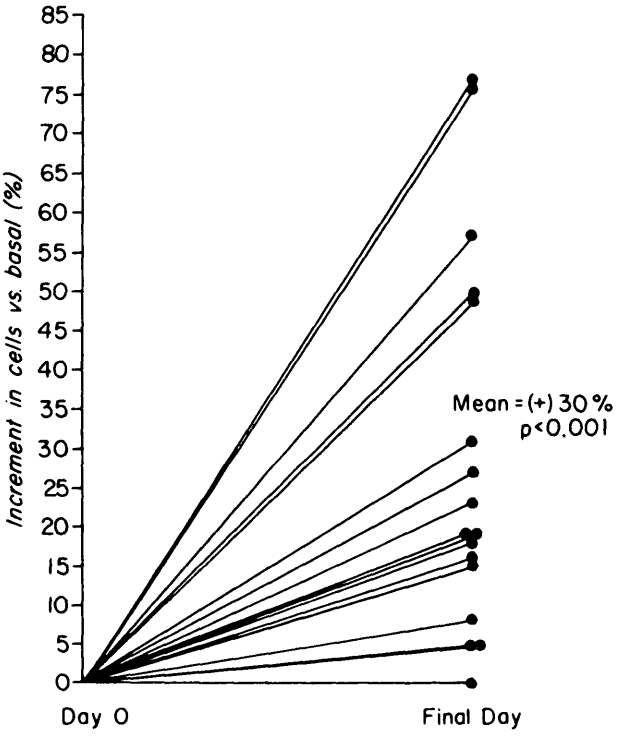

FIG. 1. Proliferation of cultured human skin fibroblasts in "high" glucose media (18.4 ± 6.5 mM). Each point represents the increment in cell number in "high" glucose media expressed as a per cent of increment in cells in "basal" medium (5.0 mM glucose) for a single cell strain in one experiment.

Substitution in two experiments of either mannitol or sucrose for sorbitol (as further controls for possible effects of hyperosmolarity) did not result in consistent enhancement of proliferation. Experiments with various sucrose concentrations resulted in changes in cell numbers of (+) 2 per cent and (−) 2 per cent at 10 mM, (−) 28 per cent and (−) 4 per cent at 20 mM, and (−) 72 per cent and (+) 24 per cent at 30 mM. The experiments with mannitol yielded negative effects on proliferation at all concentrations tested: (−) 118 per cent and (−) 65 per cent at 10 mM, (−) 118 per cent and (−) 83 per cent at 20 mM, and (−) 126 per cent and (−) 130 per cent at 30 mM.

Human arterial smooth-muscle cells were studied in a similar fashion. In contrast to the results seen with fibroblasts, no consistent effect on cell number was found in 11 experiments with nine strains using that glucose concentration (19 mM) found to be opti-

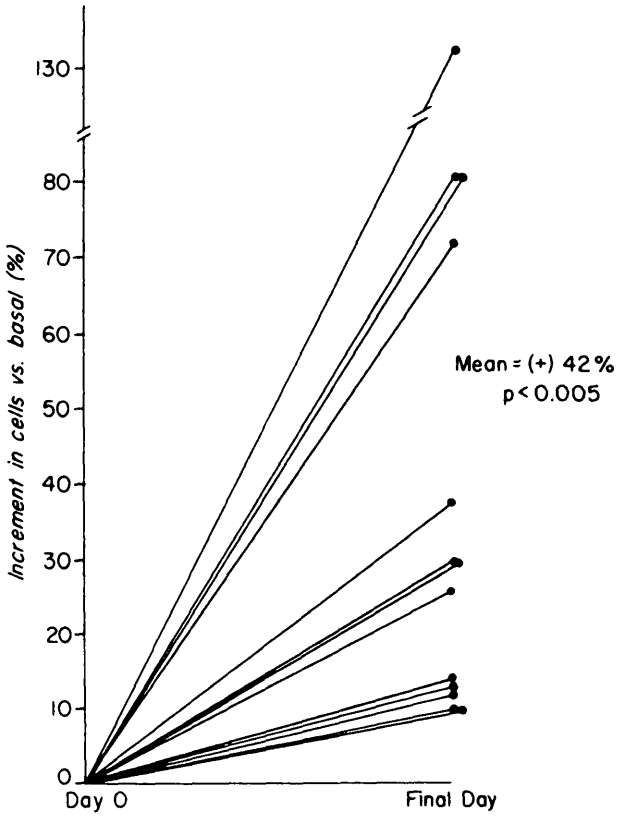

FIG. 2. Proliferation of cultured human skin fibroblasts in "high" sorbitol media (19 ± 7 mM). Each point represents the increment in cell number in "high" sorbitol media expressed as a per cent of increment in cells in "basal" medium (no added sorbitol) for a single cell strain in one experiment.

mal for promoting fibroblast proliferation (figure 3).

Experiments using added sorbitol gave similar results to those using glucose in seven experiments in which five cell strains of arterial smooth-muscle cells were used, again in contradistinction to the results with fibroblasts (figure 4). No consistent effect on smooth-muscle-cell numbers by the sorbitol concentration most effective in promoting fibroblast growth was observed.

DISCUSSION

The results of this study, indicating that "high" glucose concentrations can promote proliferation of some cell types in culture but not others, may relate to a possible connection between glucose concentrations and cell proliferation in vivo. Although "high" glucose concentrations have been used in a variety of experiments done on perfused organs or tissues,[15,16,22] there are no previously published studies that specifically focused on the effect of glucose on growth or proliferation of cultured human diploid cells in a range of glucose concentrations typically seen in many diabetic subjects. These results have shown an incremental enhancement of cell proliferation by increasing glucose concentrations to an optimum at about 18 mM (340 mg./dl.). A preliminary search was made for a possible intrinsic difference in growth behavior between cells from normal and diabetic subjects; however, none was found in the few diabetic strains tested. An exception was the behavior of two diabetic fibroblast cell strains that had been stored in liquid nitrogen for several months and thawed prior to these experiments (data not shown): they showed no response to glucose. These observations are obviously preliminary, and whether or not this effect represents a change induced by prolonged freezing of cells requires further investigation. Diabetic fibroblasts that had not been frozen, however, did not differ from normal cells in response to this particular metabolic perturbation.

The response of cells to added sorbitol, a presuma-

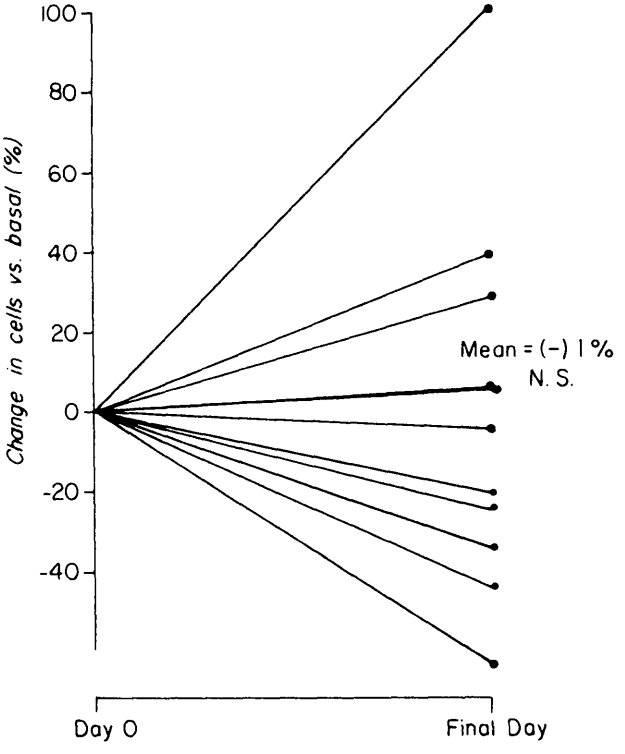

FIG. 3. Proliferation of cultured arterial smooth-muscle cells in "high" glucose media (19 ± 2 mM). Each point represents the change in cell number in "high" glucose media expressed as a per cent of increment in cells in "basal" medium (5.0 mM glucose) for a single cell strain in one experiment.

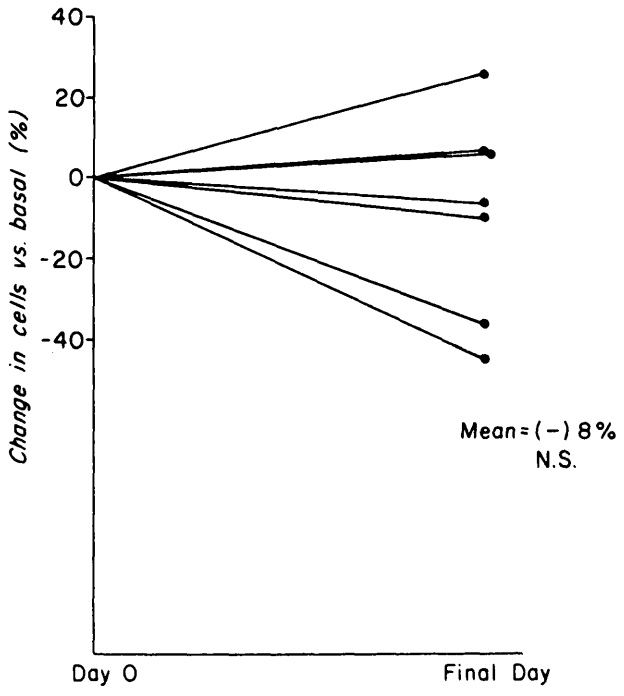

FIG. 4. Proliferation of cultured arterial smooth-muscle cells in "high" sorbitol media (18 mM). Each point represents the change in cell number in "high" sorbitol media expressed as a per cent of increment in cells in "basal" medium (no added sorbitol) for a single cell strain in one experiment.

bly nonutilizable carbohydrate, was striking, since the magnitude of proliferation enhancement was as great or greater than with glucose, and the "optimal" concentration (19 mM) was virtually identical to that for glucose. It has been held that fibroblasts are relatively "resistant" to the effects of insulin in that relatively large concentrations are required to demonstrate biologic effects in vitro.[23] However, fibroblast insulin receptors have been identified,[24] and more recent studies suggest that, under appropriate conditions, the fibroblast may be somewhat more "sensitive" to insulin than was previously thought.[25] Thus, it is conceivable that some sorbitol might be converted to fructose (and, thus, metabolized), perhaps depending on the relative magnitude of the insulin effect on the cells, but there is no direct information about this possibility.

Since the glucose concentration in the medium during sorbitol experiments was always 5 mM (90 mg./dl.), this suggests that the effect seen with "high" glucose media cannot simply be explained on the basis of greater availability of a necessary metabolic substrate. Although a partial explanation might be the facilitated glucose transport induced by hyperosmolarity, as was shown in other systems,[22] this cannot account completely for the enhanced cell multiplication, since experiments using equimolar mannitol or sucrose were without a consistent effect on cell proliferation. Possibly, with the similar concentration "optima," a similar mechanism exists for the effects produced by both glucose and sorbitol, though what that mechanism might be is obscure at this point.

Equally as intriguing is the observation that human arterial smooth-muscle cells behave differently from fibroblasts in response to both "high" glucose and to sorbitol. Great interest surrounds studies of these cells, since their central role in the pathogenesis of atherosclerosis has been recognized.[8,9] Although consistent effects on proliferation were not seen in this study, it may be that larger numbers of strains might be required to reveal significant responses. Alternatively, cells from different tissue sources might indeed respond differently to higher concentrations of glucose, as was found in this study. The one diabetic arterial smooth-muscle strain used in this study did not respond to high glucose; clearly, further strains obtained from various types of diabetic donors of diverse ages need to be tested.

It would seem important to search for possible combined effects on the proliferation and metabolism of arterial smooth-muscle cells of various concentrations of certain of the metabolic "risk factors" for atherosclerosis that are frequently found in diabetics and that can be studied in cell culture systems: hyperglycemia, high insulin concentrations, and hyperlipidemia. It has already been shown that insulin concentrations in the physiologic range can stimulate proliferation of cultured monkey arterial smooth-muscle cells.[18] Furthermore, cholesterol-rich low-density lipoproteins can enhance proliferation of monkey arterial smooth-muscle cells.[8] The metabolism of lipoproteins by cultured human arterial smooth-muscle cells is being investigated,[19,26,27] and any possible modulation of these metabolic activities by various glucose concentrations requires further study. Thus, utilization of cell culture techniques allows study of effects on proliferation and metabolism of several of the alterations in the cellular metabolic environment associated with diabetes alone or in combination. Results in the present study suggest that comparisons among different cell types and response of cells from known diabetics of various types and from presumed nondiabetics should prove fruitful.

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