High Glucose Induces DNA Damage in Cultured Human Endothelial Cells

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Abstract

Morphologic and functional abnormalities of vascular endothelium are well recognized in diabetes. In view of our previous finding that high glucose concentrations accelerate death and hamper replication of cultured human endothelial cells, we have investigated in the same model the possibility that exposure to high glucose may result in DNA damage. DNA from human endothelial cells—but not from fibroblasts—exposed to 30 mM glucose for 9–14 d manifested an accelerated rate of unwinding in alkali indicative of an increased number of single strand breaks ($P < 0.001$ vs. control). Endothelial cells exposed to high glucose also manifested an increased amount of hydroxy-urea-resistant thymidine incorporation (333±153 cpm/10^5 cells vs. 88±42 in control cells, mean±SD, $P = 0.04$), which is indicative of increased DNA repair synthesis. Neither DNA damage nor repair synthesis were increased by medium hypertonicity achieved with 30 mM mannitol. These findings suggest the possibility that, under conditions of high ambient glucose, excess glucose entry in cells that are insulin independent for glucose transport may, directly or indirectly, perturb DNA function. Further, they suggest the possibility that different individual capabilities to repair DNA damage—a process that is under genetic control—may represent a mechanism for different individual susceptibilities to development of diabetic vascular complication.

Introduction

The chain of events underlying the injuries that diabetes mellitus inflicts upon blood vessels, eventually resulting in the destructive long-term complications of the disease, is still undefined.

The vascular lesions characteristic of human and experimental diabetes suggest a primary involvement of vascular endothelium: endothelial cell morphology is abnormal (1, 2), capillary permeability is increased (3), and the amount and composition of extracellular matrix in basement membranes are altered (4). Among the many metabolic abnormalities of the diabetic milieu, hyperglycemia is the one showing in epidemiologic studies the most consistent and significant correlation with diabetic microangiopathy (5), and it has been invoked as an independent risk factor for macroangiopathy (6). Until now, however, no specific ill effect of high glucose on vascular endothelium had been identified.

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High ambient glucose is a likely potential offender for endothelial cells, since these cells are independent of insulin for glucose transport (7, 8), and thus at risk, in presence of high glucose, of intracellular accumulation of glucose, and glucose metabolites, which are well demonstrated to occur in other insulin-independent tissues when exposed to diabetic hyperglycemia (9, 10). Reducing sugars, through nonenzymatic glucosylation or oxidative reactions, may alter a host of intracellular constituents, and, among them, nucleic acids (11, 12). Our recent observation that glucose levels mimicking diabetic hyperglycemia result in excess death and decreased replication of human endothelial cells in culture (13) has led us to explore the possibility that high glucose concentrations may be toxic to endothelial cells by inducing DNA damage.

Methods

Materials. Disposable tissue culture supplies were purchased from Costar, Cambridge, MA. Medium-199, Dulbecco's modified Eagle's medium containing physiologic glucose concentration (1 g/liter), L-glutamine, penicillin, streptomycin, fungizone, and trypsin-EDTA (A-trypsin versene solution) were purchased from Irvine Scientific, Santa Ana, CA; D-glucose, from Mallinckrodt Inc., Paris, KY; and D-mannitol, from Sigma Chemical Co., St. Louis, MO. Fetal calf serum was purchased from Gibco, Grand Island, NY; epidermal growth factor (culture grade) was from Collaborative Research, Inc., Lexington, MA. All reagents employed for the study of DNA strand breaks after the procedure of Birnboim and Jevcak (14) were purchased from Sigma Chemical Co. Hydroxyurea (HU)[1] was obtained from Calbiochem-Behring Corp., La Jolla, CA. [Methyl-^{3}H]thymidine (6.7 Ci/mmol) was purchased from New England Nuclear, Boston, MA; glass microfiber filters were from Whatman Ltd., Clifton, NJ; and scintillation fluid (Beta Max) was from WestChem, San Diego, CA.

Cell culture. Human endothelial cells were obtained from umbilical veins and established in tissue culture as previously described (13). All experiments with endothelial cells were performed in primary cultures. Cells were plated in 60-mm Petri dishes at a density of 4×10^5 cells and cultured in 4 ml of Medium-199 containing 14% heat-inactivated pooled human serum and 10 ng/ml epidermal growth factor. In selected experiments, performed to evaluate whether the level of replicative activity of the cultures might affect the results of the DNA damage assay, additional dishes were seeded at higher density (1×10^6 cells). Human fibroblasts originally obtained from a baby's foreskin were used between the 9th and the 12th passage and cultured in Dulbecco's modified Eagle's medium containing physiologic glucose concentration (1 g/liter) and 10% fetal calf serum. Before use, all media were filtered and supplemented with 2 mM glutamine, 17.5 mM Hepes buffer, penicillin (50 U/ml), streptomycin (50 μg/ml), and fungizone (0.25 μg/ml). Media were changed every third day. Experimental cultures were exposed to 30 mM glucose the day after plating. To control for medium hyperosmolarity, in some experiments parallel cultures were exposed to 30 mM mannitol.

1. *Abbreviations used in this paper:* DTTP, deoxythymidine triphosphate; HU, hydroxyurea.

DNA damage. We investigated DNA damage with the fluorometric technique originally described by Birnboim and Jevcak (14) and validated by several investigators (15, 16). The assay monitors the unwinding of DNA exposed to alkali by measuring the fluorescence due to the binding of ethidium bromide to residual double-stranded DNA. The amount of double-stranded DNA remaining after a given period of alkaline denaturation is proportional to the number-average molecular weight of DNA between single strand breaks (17). For a given amount of DNA, number-average molecular weight is inversely proportional to the initial number of single strand scissions (17). Cells exposed to high glucose or mannitol for 9–14 d and respective controls were detached from the dish by trypsinization, counted, and resuspended in buffer (0.25 M meso-inositol, 10 mM sodium phosphate, 1 mM $MgCl_2$, pH 7.2) to a final density of 3.75×10^6 cells/ml. Aliquots of the cell suspension (0.2 ml) were distributed in triplicate into three sets of glass tubes: in one set (total double-stranded DNA) unwinding in alkali was prevented; in the second (background) unwinding was maximized by sonication; and in the third (experimental sample), unwinding was allowed to occur without manipulations. Cell lysis and exposure to alkali were conducted as previously described (14). At the end of the desired time of incubation in alkali (performed in the dark) cell lysates were neutralized, briefly sonicated, and diluted with 1.5 ml of the freshly prepared dye solution (ethidium bromide 6.7 μg/ml, in 13.3 mM NaOH). Fluorescence was read in a Perkin Elmer 650-10 S spectrofluorometer at an excitation wavelength of 520 nm and an emission wavelength of 590 nm. The variation among triplicates was consistently <3%. The percentage of double-stranded DNA remaining after different times of exposure to alkali was calculated by the equation (14): Percent double stranded DNA = (sample fluorescence − background fluorescence)/(total fluorescence − background fluorescence) × 100.

DNA repair. The occurrence of DNA damage most generally triggers DNA repair synthesis. We measured DNA repair synthesis as HU-resistant thymidine incorporation (18). At concentrations that markedly inhibit semiconservative DNA synthesis, HU does not appreciably affect DNA repair (19). Human endothelial cells and fibroblasts were studied after 8–15 d in culture. The medium was supplemented with 5 mM HU 1 h before addition of 28 μCi/ml methyl-[^{3}H]thymidine. 2 h after addition of labeled thymidine, monolayers were washed, trypsinized from the dishes, and cell number was determined. RNA was hydrolized with 1 N NaOH at 37°C, DNA precipitated with ice-cold 15% TCA, transferred on glass microfiber filters that were washed with 7% TCA, dried by 70% ethanol-ether and heat, and counted for radioactivity.

To exclude an effect of different intracellular deoxythymidine triphosphate (DTTP) pools on radioactive thymidine incorporation, we measured the intracellular DTTP pool in endothelial cells cultured in high and physiologic glucose concentrations. Monolayers cultured for 5–13 d were set on ice, washed, and precipitated by direct addition to the dish of chilled 6% perchloric acid. The harvested material was sonicated, centrifuged, and the supernatant was neutralized with 2 M K_2CO_3. The excess salt was removed by centrifugation and the supernatant was filtered through a 0.45-μm membrane. The filtrates were assayed for DTTP in the laboratory of Dr. M. Goulian, University of California, San Diego, employing published procedures (20).

Statistical analysis. The values for percent double-stranded DNA remaining at various time points after alkaline exposure were compared in control and high glucose cultures by analysis of variance. The values for HU-resistant thymidine incorporation in control and high glucose cultures were compared with the two tailed *t* test, paired analysis.

Results

When cultured for 9–14 d in medium containing 30 mM glucose, umbilical vein endothelial cells yielded an accelerated rate of disappearance of double-stranded DNA in alkali, which was indicative of an increased number of single strand breaks (Fig. 1). The difference between high glucose and control cultures was highly significant (F ratio, 27.0; $P < 0.001$). The effect of high glucose was not attributable to medium hyperosmolarity, since in two experiments companion monolayers cultured in the presence of mannitol (30 mM) failed to manifest increased DNA unwinding in alkali.

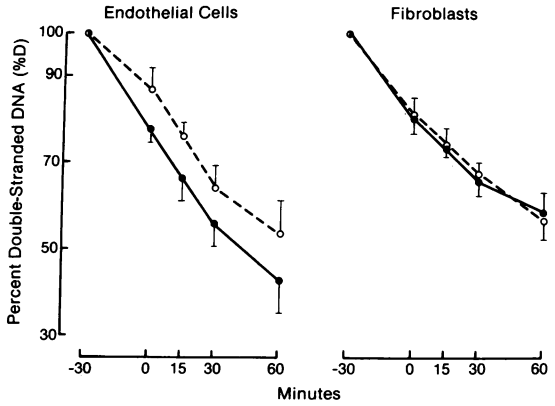

Figure 1. Kinetics of unwinding of DNA from control (○) and high glucose (●) cultures of human endothelial cells and fibroblasts. After 9 to 14 d in culture, cells were trypsinized from the dishes, counted, lysed, and exposed to alkali for the indicated length of time. Exposure to alkali was at 0°C for the first 30 min and at 15°C thereafter. The percent double-stranded DNA was calculated as described in Methods. The data represent the mean±SEM of four experiments. The difference between endothelial cells cultured in high glucose and control cells is highly significant ($P < 0.001$).

Endothelial cell cultures exposed to high glucose had consistently lower cell number, and in previous experiments (13) had shown a slightly higher proportion of cells in S phase. Since, when tested in alkaline elution, newly replicated DNA is more susceptible to alkali than bulk DNA (21), we entertained the possibility that the accelerated rate of unwinding of DNA from cells exposed to high glucose—especially in younger cultures—might reflect a possible greater amount of newly synthesized DNA. This proved not to be the case, since parallel cultures seeded at usual or double density and tested when cell number and thymidine incorporation were, respectively, reflecting active growth or saturation density, yielded superimposable results.

To identify whether endothelial cells were selectively or especially susceptible to glucose-induced DNA damage, we performed parallel studies in human fibroblasts. The DNA of fibroblasts exposed to 30 mM glucose for the same duration of time as endothelial cells (9–14 d) behaved identically to that of control cultures (Fig. 1).

Most types of DNA damage trigger DNA repair synthesis (22). We thus investigated whether cells exposed to high glucose levels exhibited increased repair synthesis by measuring thymidine incorporation in presence of concentrations of HU (5 mM) that inhibit semiconservative DNA synthesis. Endothelial cells cultured in high glucose for 8–15 d showed (Table I) a significantly increased amount of HU-resistant labeled thymidine incorporation: 333±153 cpm/10^5 cells (mean±SD) vs. 88±42 cpm/10^5 cells in control cultures ($P < 0.05$). This could not be attributed to a reduction of the intracellular DTTP pool in the high glucose cultures since their DTTP pool was 33.4±6.3 pmol/10^6 cells vs. 31.5±4.9 pmol/10^6 cells in controls (mean±SD in four experiments performed between day 5 and day 15 of culture). In fibroblasts exposed to high glucose, HU-resistant thymidine incorporation (90±16 cpm/10^5 cells) was comparable to controls (110±18 cpm/10^5 cells).

Table I. HU-resistant Thymidine Incorporation in Human Endothelial Cells Cultured in High or Physiologic Glucose Concentrations

Day of culture	5 mM glucose	30 mM glucose
	cpm/10⁵ cells	*cpm/10⁵ cells*
8	67	193
8	135	295
10	111	552
15	40	293
Mean	88	333
SD	42	153

At the indicated time in culture, the medium was supplemented with 5 mM HU 1 h before addition of 28 μCi/ml methyl-[^{3}H]thymidine (specific activity, 6.7 Ci/mmol). 2 h after addition of labeled thymidine, cells were washed, trypsinized from the dish, and counted. Thymidine incorporation into DNA was determined as described in Methods, and it was significantly higher in cultures exposed to high glucose ($P < 0.05$).

Discussion

It is unrealistic to anticipate that the complex phenomenology of diabetic angiopathy might be explained by one specific abnormality. Nevertheless, the search for events that may represent common denominators for diverse clinical end-points has merit, insofar as it may yield probes with which to systematically interrogate the pathogenetic process.

The finding that glucose levels mimicking severe diabetic hyperglycemia result in vitro in DNA damage and increased DNA repair synthesis in endothelial cells but not in fibroblasts proposes a mechanism of glucose toxicity that can be tested against a number of aspects of diabetic vascular disease and perhaps of other complications of diabetes. The levels of such testing are at least three. First, the lack of glucose toxicity for DNA of fibroblasts, which, at variance from endothelial cells (7, 8), are insulin-dependent for glucose transport (23, 24), suggests that excess unregulated entry of glucose into the cell is the primum movens for DNA damage, and thus that insulin-independent tissues would be the ones mostly affected by this form of glucose toxicity. It is unlikely that the failure to demonstrate an effect of high glucose on fibroblast DNA can be accounted for by the fact that fibroblasts were passaged whereas endothelial cells were in primary cultures. Passaged fibroblasts have been shown to maintain responsiveness to ambient glucose: they are stimulated to proliferate by high glucose (25) and they are a widely employed system for the study of insulin's glucoregulatory action (23, 24).

Second, if glucose-induced DNA damage does in fact occur in selected tissues in vivo, it could compromise their survival and their replicative capabilities. The accelerated atherogenesis plaguing diabetic patients (6) and the decreased cell density and increased argyrophilia of aortic endothelium observed in diabetic animals (2) may be expressions of compromised endothelial cells' viability. We have observed in vitro (13) accelerated death and decreased replication of endothelial cells cultured in high glucose, and although the replicative delay was mimicked by mannitol, the effect of mannitol was of significantly lesser magnitude and did not result in excess cellular death. Since mannitol failed in these experiments to induce DNA damage, it is conceivable that the greater effect of glucose on endothelial cell viability and proliferation may result from the summation of effects of hypertonicity with those of DNA damage. Other tissues might be susceptible to these effects of high glucose and especially deleterious consequences could be envisaged during embryogenesis, known to be disturbed in poorly controlled diabetic pregnancies (26).

Third, since a host of DNA lesions can be effectively repaired (22), and since repair systems are under genetic control (27) and may thus vary in efficiency among individuals, the finding of DNA damage-repair induced by high glucose introduces a possible mechanism for different individual susceptibilities to the vascular complications of diabetes (28, 29).

The diabetic state is not to our knowledge accompanied by an increased incidence of neoplasia. This implies that, if occurring in vivo, DNA lesions induced by high glucose do not result in important chromosome aberration. They might, however, be sufficient to alter gene expression and perhaps viability of certain cellular systems, thus resulting, if severe hyperglycemia is sufficiently protracted, in a host of abnormalities of structure and function. Further studies investigating the nature and kinetics of the effects of high glucose on DNA and mostly whether such effects occur in vivo are warranted.

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