High glucose inhibits human epidermal keratinocyte proliferation for cellular studies on diabetes mellitus

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ABSTRACT

In order to more clarify the delayed wound healing in diabetes mellitus, we cultured the human epidermal keratinocytes in both 6 mM (control group) and 12 mM glucose (high-glucose group) of 'complete' MCDB 153 medium. Hyperglycaemia slowed the rate of their proliferation and inhibited their DNA synthesis and the production of total proteins. By 1 month after primary seeding in high-glucose group, the cells ceased their proliferation, whereas the cells in control group grew for more than 40 days. Mean population doublings in high-glucose group was 5·27 (vs. 7·25 in control, $P = 0{\cdot}001$), and mean population doubling time during 1 month in high glucose group was 5·43 days (vs. 3·65 days in control, $P = 0{\cdot}02$). They indicate that prolonged exposure to high glucose decreases the replicative life span of human epidermal keratinocytes *in vitro*. Furthermore, analysis of fatty acid contents in membrane phospholipids with thin-layer and gas chromatography showed no difference between the cultured keratinocytes in both conditions. Immunocytochemical staining of glucose transporter 1 shows that 28·1% of cells in high-glucose group were almost twice positive of those in control group (13·2%, $P = 0{\cdot}008$). The mechanism of the ill effects of high glucose on epidermal keratinocytes is not so far clear, but it indicates the possibility of any direct effect of hyperglycaemia on glucose metabolism without changing lipid metabolism on cell membrane. The high-glucose group presented in this report can be available as an *in vitro* valuable study model of skin epidermal condition on diabetes mellitus.

Key words: Diabetes mellitus ● Epidermal keratinocytes ● Glucose ● Lipids ● Wound healing

Key Points

- diabetes mellitus exhibits various metabolic abnormalities at tissue level
- it is known so far that high glucose inhibits cell proliferation in human endothelial cells and fibroblasts but to date there is no report about the effect of high glucose on human epidermal keratinocytes

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INTRODUCTION

In diabetes mellitus, various metabolic abnormalities occur at tissue level. Among their complications, we often meet and suffer from many delayed wound healing situations that include vascular disorders, incapability of wound contraction and disturbing epithelialisation (1). Although it is known that high glucose inhibits cell proliferation in human endothelial cells (2–4) and fibroblasts (5), there is no report about the effect of high glucose on human epidermal keratinocytes. It is important to determine in diabetes mellitus, a disease characterised by delayed wound healing, especially disturbing epithelialisation, whether the hyperglycaemia of diabetes mellitus is a likely

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potential offender for human epidermal keratinocytes. In order to more clarify its mechanism, we used the culture system of human epidermal keratinocytes in this report. It is very interesting to determine the influence of an excessive glucose level on the skin epidermal keratinocytes in culture system. All cell-culture media must contain a sugar or closely related substance as the major energy source. Glucose is the most widely used sugar (6–8). At present, a low-calcium serum-free medium (MCDB 153) is most commonly used for clonal growth of human epidermal keratinocytes (8). MCDB 15X series contains glucose at 6 mM (about 1 mg/ml), the concentration to which mammalian tissues are normally exposed (9). To study the effects of high glucose on endothelial cells and fibroblasts *in vitro*, a 20 mM glucose concentration was chosen to match glucose levels in uncontrolled and non ketotic diabetic patients (2–5). It is very difficult to decide the optimal glucose concentration of diabetic condition for epidermal keratinocytes in culture, because there is a kind of barrier, so-called basement membrane, between skin epidermis and dermis. So, we thought a smaller concentration than 20 mM was closely optimal for skin epidermal condition of diabetic patients and set high-glucose condition tentatively at twice (12 mM) of normal glucose condition. Recently, several types of glucose transporter (GLUT1–7), specific plasma membrane glycoproteins, have been reported at the level of various barriers of mammalian tissues (10). In skin, GLUT1 is highly expressed in the basal layer of human epidermis (11,12). Although the detailed function of GLUT1 is not known in the epidermis, an increase in GLUT1 expression has been described as a high metabolism of glucose (13). Therefore, we studied the immunocytochemical staining of GLUT1-antibody on the cultured epidermal keratinocytes in normal and high-glucose conditions. Furthermore, we analysed the total fatty acid composition in membrane phospholipids of the cultured keratinocytes by using gas and thin-layer chromatography (GC and TLC), in order to study whether high glucose has any influence on lipid metabolism of cell membrane phospholipids that strongly related with cell differentiation and proliferation (14–21).

METHODS

Materials

Amino acids and medium reagents for MCDB 153, fetal bovine serum (FBS), D-(+)-glucose, trypsin type IX, trypsin inhibitor type I-S and gentamicin (10 mg/ml) were obtained from Sigma (St Louis, MO, USA). Plastics (T-75 flask) were from Laboratory Science Co. (Corning, NY, USA). Methanol and chloroform were obtained from B&J Baxter (McGraw Park, IL, USA). Petroleum ether (30–60°C) and benzene were purchased from Baker (Phillipsburg, NJ, USA). Frozen bovine pituitaries were from Pel-Freeze (Rogers, AR, USA) and filters were obtained from Millipore, Corp. (Bedford, MA, USA). The 250-μm mesh filter was obtained from PGC Scientific (Gaithersburg, MD, USA). Chelex 100 Cheleting Ion Exchange Resin was from Biorad (Hercules, CA, USA) for making chelex FBS. Primary antibody of glucose transporter 1 (GLUT1) was from Chemicon (Temecula, CA, USA). Avidin–biotin–peroxidase complex was from Vector Laboratories (Burlingame, CA, USA).

For gas chromatography 18 fatty acid standards and 17:0 (Heptadecanoic acid) were obtained from Nu-CHEK-PREP, Inc. (Elysian, MN, USA). Thin-layer silica gel 60 plates were from Merck (Darmstadt, Germany).

Primary cell culture

Bovine pituitary extract (BPE) is a 0·15 M NaCl homogenate of frozen powdered pituitaries that was clarified by ultracentrifugation twice at 200 000 × *g* and filtered through 0·8-, 0·45- and sterile 0·22-μm filters. The basic medium, MCDB 153, was prepared as described by Boyce and Ham (8) and was supplemented with $0{\cdot}6 \times 10^{-6}$ M (0·218 μg/ml) hydrocortisone, 5 ng/ml of epidermal growth factor, 5 μg/μl of insulin, 6 mg% BPE and 0·15 mM $CaCl_2$ to form MCDB 153 complete medium (glucose concentration: 6 mM as normal control medium). Human epidermis was obtained from fresh surgical resection specimens from mammary reduction operations of seven females (age range: 16–46 years, average: 31·9 years). To obtain skin epidermal cells, the skin samples were cut into small strips without deep dermis and subcutaneous tissue and then digested overnight at room temperature with 0·07% trypsin in solution A (30 mM hydroxyethylpiperazine-*N*′-2-

Key Points

- high levels of glucose were evaluated in the culture system for human epidermal keratinocytes
- the immunocytochemical staining of GLUT1 antibody on the cultured epidermal keratinocytes was evaluated at normal and high glucose conditions
- we analysed the total fatty acid composition in membrane phospholipids of the cultured keratinocytes using GC and TLC

ethanesulphonic acid, 10 mM glucose, 3 mM KCl, 130 mM NaCl, 1·0 mM Na_2HPO_4, pH 7·4). The tissue was then separated at the epidermal–dermal junction, leaving the basal cells on the dermal layer. The basal cells were then gently scraped from the dermis into MCDB 153 complete medium plus 10% FBS. The cell suspension was centrifuged. The filtered cell suspension was counted with haematocytometer and plated at 20×10^6 cells in 15 ml of MCDB 153 complete medium containing 2% chelexed FBS per T-75 flask and incubated at 37°C in 5% CO_2 (22,23).

Subcultivation

High-glucose medium (glucose concentration: 12 mM) was made by adding 560 mg of D-(+)-glucose in 500 ml of the basic medium MCDB 153 and was sterile filtered. The cell cultures were fed every 48 hours until almost 70% confluence was reached. The cells were passaged by incubation at 37°C with 0·03% trypsin−0·01% EDTA and plated at 2×10^6 cells per T-75 in MCDB 153 complete medium. On the next day, the flasks were divided into two groups–with normal glucose medium (control medium) and high-glucose medium (glucose concentration: 12 mM). Cells were passaged every 4–5 days. At each passage, cells were counted, and the other keratinocyte cultures in flasks were rinsed twice with calcium-free, phosphate-buffered saline (PBS) and were fixed by 1 ml of methanol into —80°C freezer until use. Cells were cultured until the cell number decreased.

Indices of cell growth

Both seven-keratinocyte strains were obtained. Population doubling (PD) and population doubling time (PDT) during 1 month were calculated as previously described (24). The cumulative total number of cells at each passage was determined, and the total number of cells in control group at each passage represents 100% compared with that in high-glucose group. The protein and DNA were processed for modified Lowry protein (25) and Burton DNA (26) as described (22,27), and the cumulative total amounts were calculated to compare two groups.

Immunocytochemistry

Each sample of subculture P4 was rinsed with PBS and fixed with 4% paraformaldehyde at room temperature. The dishes were first incubated with normal goat serum for 20 minutes to prevent non specific antibody binding and were incubated with primary antibody of GLUT1 (1:250) for 24 hours at 4°C and then incubated with biotinylated goat anti-rabbit immunoglobulin G for 30 minutes. Avidin–biotin–peroxidase complex was applied for 45 minutes. A solution of peroxidase substrate 3,3′-diaminobenzidine tetrahydrochloride was used to visualise the resulting complex. The percentage of immunopositive cells for GLUT1 was determined at ×100 magnification by counting cells from three randomly selected fields.

Lipid analysis

Cells of subculture P4 were extracted with a 1:2:1·5 ratio of methanol : chloroform : 0·1 M KCl in 50% methanol, and the organic phase was re-extracted with ×2·5 volume of 0·1 M KCl in 50% methanol. The extracted fraction was suspended in 75 µl of 1:1 chloroform : methanol after evaporating under a nitrogen stream, applied to a TLC plate and chromatographed in one direction using chloroform : methanol : glacial acetic acid (90:8:1). The fatty acid composition of the cellular phospholipid fraction reflects the percentage of fatty acid amounts of all the fatty acid-containing lipids in the cells. Therefore, all our lipid analyses were performed on the TLC-separated phospholipid fraction. After TLC chromatography of the cell lipids, the phospholipid-containing area of the plate was scraped and material was eluted from the silica during transmethylation with 6% methanolic HCl. Fifty micrograms of 17:0 was added at this time (Heptadecanoic acid) as an internal standard, and the sample was heated for 3·5 hours at 80°C in order to form fatty acid methyl esters (FAMEs). The FAMEs were resuspended in 200 µl of benzene, filtered using a 0·45-µm filter, evaporated and resuspended in 100–150 µl (as determined by the quantity of protein) of filtered chloroform for analysis. Half microlitre of chloroform was injected for analysis using a Shimazu gas chromatograph (GC) model GC-14A equipped with a J and W Scientific (Folsom, CA, USA) fused silica Megabore DB225, 0·53-µm diameter column. The FAMEs were eluted with scrubbed helium at a flow rate of 2·79 ml/minute at 210°C for 16 minutes, at a

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gradient of 4°C/minute until 220°C and then isothermic until 18·5 minutes. The flame ionisation detector output of the gas chromatograph was digitised by an IBM-PC computer interface (model AN-146, Alpha Products, Darien, CT, USA). Both the recording and data evaluation software were written in BASIC or FORTRAN (21,23,28).

RESULTS

Cell morphology

Figure 1 shows normal clonal epidermal keratinocytes with colony formation in control medium. Figure 2 shows that there is a small group of large and flattened cells in the centre of the colony in high-glucose medium.

Cell growth

By 1 month after primary seeding in high-glucose group, the cells ceased their proliferation, whereas the cells in control group grew for more than 40 days. The cumulative total number of cells in high-glucose medium at each passage was compared with that in control medium at each passage represented as 100% using a two-tailed Student's *t*-test. The cells in high-glucose medium grew well until subculture P2, but gradually ceased proliferation. After subculture P6, there was a significant difference between the two groups (Figure 3). Mean population doubling (MPD) and mean population doubling time (MPDT) were calculated during the first month (Table 1). The comparison was made only within subcultures established from the same primary culture. The values of MPD and MPDT in high-glucose medium were compared with those in control medium using a two-tailed Student's *t*-test. MPD in high-glucose group was 5·27 (vs. 7·25 in control, $P = 0·001$), and MPDT in high-glucose group was 5·43 days (vs. 3·65 days in control, $P = 0·02$).

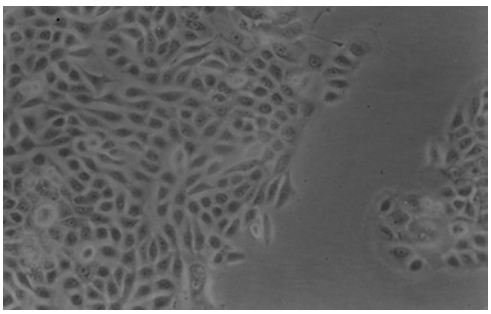

Figure 1. The photograph shows the cultured epidermal keratinocytes of subculture P2 in control medium (glucose concentration: 6 mM). The clonal cuboidal cells grow well with colony formation (magnification, ×100).

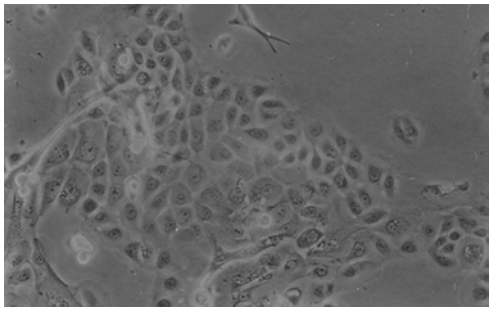

Figure 2. The photograph shows the cultured epidermal keratinocytes of subculture P2 in high-glucose medium (glucose concentration: 12 mM). There is a nest of large and fattened cells into the colony (magnification, ×100).

Total DNA synthesis and total proteins

The cumulative total amount of DNA synthesis and proteins in high-glucose medium at each passage were compared with those in control medium at each passage represented as 100% using a two-tailed Student's *t*-test (Figures 4 and 5). Both trends were parallel to that of total cell number (Figure 3). After subculture P5, there were significant differences between the two groups (Figures 4 and 5).

Immunocytochemical findings of GLUT1

The immunocytochemical staining for GLUT1 in the cells was in general confined to the cytoplasm

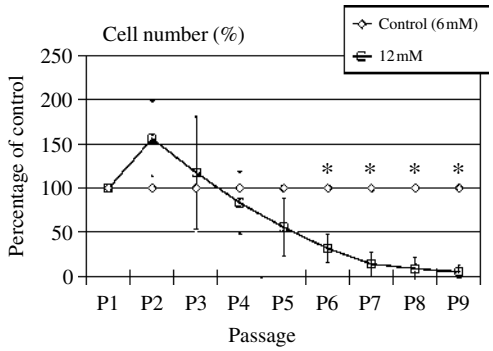

Figure 3. The cumulative total cell number in high-glucose medium at each passage are compared with those in control medium at each passage, represented as 100%, using a two-tailed Student's ***t***-test. After subculture P6, there is a significant difference between the two groups (*$P < 0·001$).

Key Points

- one month after primary seeding in high glucose group, the cells ceased their proliferation, whereas the cells in control group grew for more than 40 days

Key Points

- we have demonstrated that human epidermal keratinocytes cultured in twice concentrations of glucose are substantially delayed in reaching confluence
- our data indicate that prolonged exposure to high glucose decreases the replicative life span of human epidermal keratinocytes culture

Table 1 Mean population doubling (MPD) and mean population doubling time (MPDT)of the cultured epidermal keratinocytes in normal and high-glucose group

Group	*n*	MPD	MPDT (days)
6 mM (normal glucose)	7	7·25	3·75
12 mM (high glucose)	7	5·27	5·43
Statistical assessment		$P = 0·001$	$P = 0·02$

of cells. We counted cells per field on average 321 in normal group (10·4–15·2%) and 179 in high-glucose group (23·3–32·4%). The percentage of GLUT1-expressed cells was 13·2% on average in normal group, whereas it was almost double (28·1%) in high-glucose group (Table 2).

Total fatty acid composition in membrane phospholipids

Gas chromatograms of the cultured cells showed no difference between control and high-glucose groups. The chromatography demonstrated a typical chromatogram pattern of the keratinocytes cultured in serum-free and essential fatty acid-free medium. It showed a large increase of monounsaturated fatty acids, little polyunsaturated fatty acids and a small reduction of saturated fatty acids previously described (17,21,23).

DISCUSSION

We have demonstrated that human epidermal keratinocytes cultured at twice the standard concentration of glucose are substantially delayed in reaching confluence. The 20 mM glucose concentration has been so far chosen to match glucose levels in uncontrolled and non ketotic diabetes mellitus for the cultured fibroblasts and vascular endothelial cells (2–5). For culturing the keratinocytes, what concentration of glucose is optimal to match actual diabetic condition is not important. One of the reasons is whether the skin epidermis is exposed to normal glucose level (6 mM), like other mammalian tissues, is not clear because of the existence of the barrier (basement membrane) between epidermis and dermis. The other reason is that the glucose concentration per flask is not stable, because each cell number is easily changed. Actually, the cell number in high-glucose group is smaller than that in control group (Figure 3). So, the glucose concentration per cell in high-glucose group becomes higher, sooner or later. The important thing is whether high glucose is toxic for epidermal keratinocytes. Our data indicate that prolonged exposure to high glucose levels decreases the replicative life span of human epidermal keratinocytes *in vitro*. The epidermis is not directly vascularised, so basal cells should supply glucose to the whole epidermis by GLUT1 (13). Although our data do not show the toxic effects of glucose occur *in vivo*, high expression of GLUT1 induced by

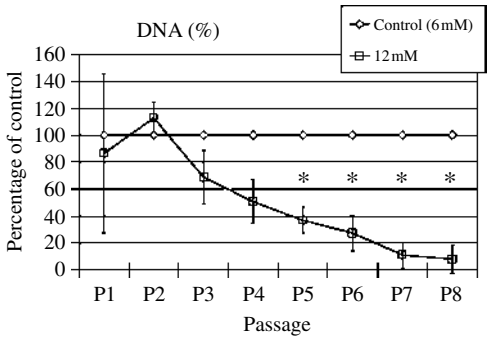

Figure 4. The cumulative total amounts of DNA synthesis in high-glucose medium at each passage are compared with those in control medium at each passage, represented as 100%, using a two-tailed Student's ***t***-test. After subculture P5, there is a significant difference between the two groups (*$P < 0·001$).

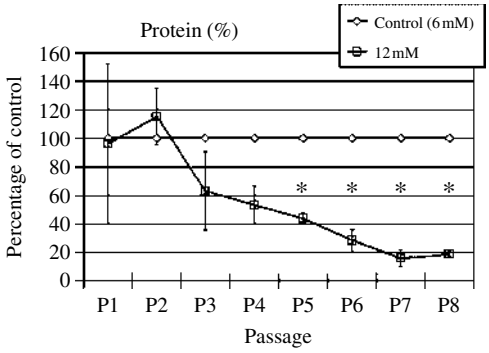

Figure 5. The cumulative total amounts of protein in high-glucose medium at each passage are compared with those in control medium at each passage, represented as 100%, using a two-tailed Student's *t*-test. After subculture P5, there is a significant difference between the two groups (*$P < 0·001$).

Table 2 Percentage of positively stained cells by glucose transporter 1-antibody immunocytochemically in normal and high-glucose group

Group	*n*	Percentage
6 mM (normal glucose)	3	13·2
12 mM (high glucose)	3	28·1
Statistical assessment		$P = 0·008$

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high glucose *in vitro* (Table 2) might render the other keratinocytes in suprabasal layers exposed to high-glucose condition *in vivo*. In cell types not damaged by hyperglycaemia, like vascular smooth muscle cells (29), skeletal muscle cells (30) and adipocytes (31), elevated extracellular glucose concentration has been shown to downregulate glucose transporter, as a protective mechanism. In cell types damaged by hyperglycaemia, like vascular endothelial cells, increased extracellular glucose concentration accelerated the rate of glucose transport without changing in the expression of GLUT1 (29,32). So, the reaction for hyperglycaemia in epidermal keratinocytes may be close to that in vascular endothelial cells. By contrast, the epidermal keratinocytes *in vitro* can synthesise *de novo* fatty acids in the conversion of glucose by glycolysis (28). The fatty acids of cellular phospholipids, which are critical structural components of cellular membranes, are strongly related with the proliferation and differentiation of the keratinocytes (14–21). We also demonstrated that high glucose did not have any influence on total fatty acid composition of cellular phospholipids of the cultured keratinocytes. It indicates the possibility of any direct effect of hyperglycaemia on glucose metabolism without changing lipid metabolism on cellular membranes. In conclusion, the high-glucose group presented here can be a good model to study the delayed epithelialisation on diabetes mellitus, using cultured epidermal keratinocytes. We have not examined the migration study of the keratinocytes in both the conditions, which is important to wound repair process. The migration of the epithelial cells from areas adjacent to the injury, a process called restitution, comprises rapid closure by an actinomyosin (33). But this event is regulated by the crosstalk of growth factors, integrins and metalloproteases between the epidermal keratinocytes and the mesenchymal cells (34,35). The importance of further study is related to the migration abilities in both conditions using the three-dimensional composite skin model.

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Key Points

- we have also demonstrated that high glucose did not have any influence on total fatty acid composition of cellular phospholipids of the cultured keratinocytes
- it indicates the possibility of any direct effect of hyperglycemia on glucose metabolism without changing lipid metabolism on cellular membranes
- in conclusion, the high-glucose group presented here can be a good model to study the delayed epithelialisation on diabetes mellitus, using cultured epidermal keratinocytes

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