High glucose and endothelial cell growth: novel effects independent of autocrine TGF-β1 and hyperosmolarity

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1Department of Medicine, Kolling Institute: Renal Research Group, and 2Department of Immunology, Royal North Shore Hospital, St Leonards, 2065; and 3Department of Physiology, University of Sydney, New South Wales 2006, Australia

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McGinn, S., P. Poronnik, M. King, E. D. M. Gallery, and C. A. Pollock. High glucose and endothelial cell growth: novel effects independent of autocrine TGF-β1 and hyperosmolarity. Am J Physiol Cell Physiol 284: C1374–C1386, 2003—Small increases in capillary glucose inhibit endothelial cell proliferation and cell cycle progression, and the effects of TGF-β1 and TGF-β neutralization on these parameters were studied. High glucose and hyperosmolarity increased endothelial cell TGF-β1 secretion (P < 0.0001) and bioactivity (P < 0.0001). However, high glucose had a greater effect on reducing endothelial cell number (P < 0.001) and increasing cellular protein content (P < 0.001) than the osmotic control. TGF-β antibody only reversed the antiproliferative and hypertrophic effects of high glucose. High glucose altered cell cycle progression and cyclin-dependent kinase inhibitor expression independently of hyperosmolarity. High glucose increased endothelial cell apoptosis (P < 0.01), whereas hyperosmolarity induced endothelial cell necrosis (P < 0.001). TGF-β antibody did not reverse the apoptotic effects observed with high glucose. Exogenous TGF-β1 mimicked the increased S phase delay, but not endoreduplication observed with high glucose. High glucose altered endothelial cell growth, apoptosis, and cell cycle progression. These growth effects occurred principally via TGF-β1 autocrine pathways. In contrast, apoptosis and endoreduplication occurred independently of this cytokine and hyperosmolarity.

endothelial cells; diabetes mellitus; apoptosis; endoreduplication; transforming growth factor-β1

DIABETES MELLITUS contributes significantly to the morbidity and mortality within the developed world largely as a consequence of its effects on both the micro- and macrovasculature. A number of the complications of patients with diabetes are manifestations of aberrant cell growth such as the renal hypertrophy of early diabetes, the impaired wound healing from delayed capillary morphogenesis, and proliferative diabetic retinopathy. Inhibition of endothelial cell proliferation by high glucose has been documented in a number of in vivo and in vitro studies; however, the mechanisms by which these growth patterns occur has not been fully delineated (24, 42, 43).

Previous studies have demonstrated that long-term exposure (7–14 days) to high glucose leads to a reduction in endothelial cell proliferation and a concomitant increase in apoptosis (2, 8, 9, 13, 19, 25, 26, 37). Although the precise mechanisms that mediate these effects remain unclear, two lines of evidence suggest that TGF-β1 may mediate the effects of high glucose: 1) TGF-β1 has been shown to induce changes in the growth of endothelial cells similar to those observed after exposure of endothelial cells to high glucose (30, 31, 45, 52), and 2) it has been demonstrated in several cell types that exposure to high glucose is a potent inducer of TGF-β1 production (21, 38). Furthermore, increased levels of TGF-β1 are observed in both clinical and experimental models of diabetes (39, 41), and as a result, the deleterious effects of high glucose are attributed primarily to the autocrine action of TGF-β1. In mesangial cells, TGF-β1 has been demonstrated to induce its growth effects by causing arrest in the cell cycle by increasing the expression of the cyclin-dependent kinase inhibitors (CDKIs) p21Cip1 and p27Kip1 (39, 40). There is, however, little evidence establishing a direct link between the effects of high glucose on growth, cell cycle progression, and apoptosis and the autocrine actions of TGF-β1 in endothelial cells. It remains contentious whether exposure to high glucose does induce TGF-β1 production by endothelial cells, with one study showing an increase in TGF-β1 production (34) and the other study failing to show such an increase (5).

The role that the increase in osmolarity associated with high glucose may play on autocrine TGF-β1 secretion has not been fully defined. If autocrine TGF-β1 does mediate endothelial cell growth effects induced after high glucose exposure, the relative role of hyperosmolarity plays needs to be elucidated. In murine mesangial cells, exposure to high glucose increased TGF-β1 bioactivity and protein production, and this was independent of any associated increase in osmolarity (15). However, the effects of the increased osmolarity of high glucose on endothelial cell TGF-β1 secre-
tion, and hence TGF-β1-mediated growth, has not previously been investigated.

Several studies demonstrate that high glucose induces hypertrophy in a number of cell types, but whether endothelial cell hypertrophy occurs under these conditions has not been previously demonstrated. Furthermore, while others have shown similarities in cell growth responses as a consequence of the minor increase in osmolarity with high glucose and its effect on Na+/H+ antiporter activity (1), no previous study has dissociated the glucose specific growth effects from either those induced by the increase in osmolarity itself or the changes in cytokine profile that this minor increase in osmolarity induces. Changes in cell volume are known to have profound effects on cellular homeostasis. Cell swelling inhibits proteolysis and increases protein synthesis, whereas cell shrinkage stimulates proteolysis and decreases protein synthesis (22). These effects on protein metabolism may be partly mediated by alterations in the activity of lysosomal enzymes as a result of pH changes (4). Importantly, part of the hypertrophic effect previously demonstrated to occur with TGF-β1 may be due to the activation of Na+/H+ exchange (18), leading to cell swelling and alkalization with subsequent reduction in proteolysis (47). Hence, the hypertrophy that is observed in a number of cell types after high glucose exposure may be as a consequence of alterations in cell volume with resultant effects on TGF-β1. Cell volume changes may also influence cell death mechanisms, with cell shrinkage being a hallmark of apoptosis (22). Apoptosis has been shown to be increased after high glucose exposure to endothelial cells (2), but the relative contribution of TGF-β1 and hyperosmolarity to this increase in cell death is not known.

Exposure of endothelial cells to high glucose has at least three broad components, those attributed to the specific metabolic effects of increased levels of glucose such as reactive oxidative stress, those due to the hyperosmotic component of high glucose medium, and those due to the autocrine release of cytokines such as TGF-β1 production. Furthermore, whether TGF-β1 is a common mediator for the effects of high glucose and increased osmolarity on endothelial cell growth and apoptosis needs clarification. Consequently, the aim of the current study was to define the precise contribution of TGF-β1 and hypertonicity that is induced by high glucose on endothelial cell growth and progression through the cell cycle.

**METHODS**

**Cell Isolation and Culture Conditions**

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords of normal pregnancies by the method of Jaffe et al. (16). Ethical approval for the study was obtained from the Royal North Shore Hospital Human Research Ethics Committee. These cells were cultured on 0.2% gelatin (BDH, Poole, UK)-coated flasks in medium M199 (ICN, Aurora, OH) supplemented with 10% fetal calf serum (GIBCO BRL, Gaithersburg, MD), penicillin (100 U/ml), streptomycin (100 µg/ml; GIBCO BRL), heparin (100 µg/ml; Sigma, St. Louis, MO), and endothelial cell growth supplement. Endothelial cell growth supplement was obtained from bovine hypothalamus (27) and tested against a commercial preparation (Sigma). Cells were grown at 37°C in a humidified 5% CO2 incubator and subcultured at confluence by trypsinization with 0.05% trypsin-0.02% EDTA (GIBCO BRL). These cell cultures were confirmed to be endothelial on the basis of their characteristic cobblestone morphology under phase-contrast microscopy and by staining positive for von Willebrand factor using standard immunoperoxidase methods (DAKO, Carpenteria, CA).

HUVECs are a commonly used model in the study of diabetic vascular disease (2, 5, 9, 25, 26, 37, 51). They were studied between passages 1 and 4 (in which both we and others have demonstrated no phenotypic change; Ref. 10). In all experiments the cells were grown on 0.2% gelatin-coated plates to 90% confluence (days 6–8 after plating) and then exposed to medium containing 5 mM D-glucose (control), 25 mM (high) D-glucose, or 20 mM mannitol with 5 mM D-glucose (osmotic control) for 72 h. In subsequent experiments, endothelial cells were exposed to 5 mM glucose plus TGF-β1 (1 ng/ml) (Sigma) or panspecific TGF-β antibody (30 µg/ml) (R&D Systems, Minneapolis, MN), with or without high glucose, for 72 h. The medium was changed every 48 h.

**Total TGF-β1 Production**

Total TGF-β1 released into the supernatant by endothelial cells was measured using a TGF-β1 ELISA kit (Promega, Madison, WI). Confluent cells were grown on 96-well plates and exposed to the defined experimental conditions for 72 h. The supernatants were collected at 72 h, 50-µl aliquots were acid treated to activate the TGF-β1 present, and the ELISA was performed on these samples.

**TGF-β1 Bioactivity**

βig-h3 is a novel 63-kDa TGF-β1-induced gene product that has been used as a downstream indicator of TGF-β1 bioactivity (11, 20, 23, 33). The upregulation of βig-h3 expression after endothelial cell exposure to TGF-β1 (1 ng/ml) was confirmed by Western blot (data not shown). The specificity of this antibody was assessed by O’Brien et al. (33). Western blotting was used to measure the levels of βig-h3 induced under the various experimental conditions. Cells were grown on 48-well plates (Costar, Cambridge, MA) and then exposed to the different treatments. After 72 h, Triton X-100-soluble fractions were prepared from cells using 50 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100, and protease inhibitors (Roche Diagnostics, Mannheim, Germany) and were centrifuged at 10,000 rpm for 4°C for 10 min. The Triton X-100-soluble fractions were then mixed with 6× Laemmli gel sample buffer containing mercaptoethanol and heated to 95°C for 10 min. Samples were run on 7.5% SDS-polyacrylamide gel and then transferred to a Hybond nitrocellulose filter (Amersham Pharmacia Biotech, Amersham, UK) by Western blotting. The blot was blocked overnight with 5% casein in PBS and 0.1% Tween 20. After thorough washing, the blot was incubated for 2 h at room temperature with an anti-βig-h3 polyclonal antibody (1:2,000; obtained from R. E. Gilbert, St. Vincent’s Hospital, Fitzroy, Victoria, Australia). After the blot was washed four times with PBS and 0.5% Tween 20, horseradish peroxidase-labeled secondary anti-rabbit antibody (Amersham Pharmacia Biotech) was added at a dilution of 1:2,500, and the blot was incubated for a further 2 h at room temperature. The blot was then developed using the Amersham enhanced chemiluminescence kit (ECL; Amersham Pharmacia Biotech) and visualized with X-ray film. The re-
sultant films were scanned into a computer, and the relative band intensities were measured using the NIH Image software. Because equal volumes of sample were loaded onto each gel, analysis of the band intensities was then adjusted to take into account the differences in cell number observed with each treatment.

Growth Studies

To determine the effects of exposure to high glucose on cell growth, we determined cell number as a measure of cell proliferation and total cellular protein content as a measure of hypertrophy. Flow cytometry was used to analyze progression through the cell cycle. Endothelial cells were grown in 96-well tissue culture plates (Costar), and measurements were performed at 24-h intervals over a 72-h period. Cell number was measured by counting trypsinized cells with a hemocytometer. Total cellular protein content was determined on cell lysates using the Bio-Rad protein assay dye (Hercules, CA). Cells were washed in PBS and solubilized in 0.2 M sodium hydroxide, and protein content was determined per the manufacturer’s instructions.

Cell Cycle Analysis

For cell cycle analysis, endothelial cells were grown on 6-well plates and, after 72 h of exposure to the glucose-specific medium, harvested by trypsinization. Cells for pre-G1 peak analysis were fixed in 70% ethanol, whereas cells for cell cycle analysis were not fixed. The cells were then washed once in PBS and incubated for 1 h at 4°C in 1 ml of a fluorochrome solution containing propidium iodide (PI; 50 μg/ml), RNase A (1 mg/ml), and 1.5% Triton X-100 (all from Sigma) in PBS. Flow cytometry was performed on the cells using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The PI fluorescence of individual nuclei and the forward and side scatter were all measured using identical instrument settings with a minimum of 20,000 events.

\[ p21^{Cip1/Waf1} \] and \[ p27^{Kip1} \] Expression

Because previous investigations have demonstrated a delay in cell cycle traversal in the S phase after exposure of high glucose to endothelial cells (26), the expression of the TGF-β1-inducible CDKIs that induce S phase delay were measured.

The expression of \[ p21^{Cip1/Waf1} \] and \[ p27^{Kip1} \] was measured by Western blotting using the method described for Big-h3. The primary antibodies anti-\[ p21^{Cip1/Waf1} \] and anti-\[ p27^{Kip1} \] (Upstate Biotech, Lake Placid, NY) were used at a concentration of 1 μg/ml. Secondary antibody concentrations were used as anti-mouse antibody (1:1,000) for \[ p21^{Cip1/Waf1} \] and anti-rabbit antibody for \[ p27^{Kip1} \] (Amersham Pharmacia Biotech). The resultant films were scanned into a computer, and the relative band intensities were measured using the NIH Image software. Because equal volumes of sample were loaded onto each gel, analysis of the band intensities was then adjusted to take into account the differences in cell number observed with each treatment.

Cellular Apoptosis

All apoptotic parameters were assessed on adherent cells, and hence, the levels of apoptosis measured may underestimate the total levels of apoptosis that occur in vitro. However, because the cell supernatants would contain a number of apoptotic cells that may have undergone secondary necrosis, use of nonadherent cells would make interpretation of results difficult.

The terminal stages of apoptosis were determined by morphological assessment using 4,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) staining (Sigma). Cells were grown on 1-cm gelatin-coated glass coverslips, exposed to glucose-specific medium for 72 h, fixed in ice-cold methanol, and then stained with DAPI (0.8 μg/ml PBS) for 1 min at room temperature. The coverslips were mounted on slides with Glycergel mounting medium (DAKO), and the DAPI-stained nuclei were visualized with a fluorescence microscope (Olympus BX60) with a ×60 objective by using 360-nm excitation and 460-nm emission wavelengths. Apoptotic cells were defined as those clearly showing chromatin condensation, nuclear fragmentation, or the formation of apoptotic bodies. A total of 400 cells was counted per experimental treatment group, and the numbers of apoptotic cells were then expressed as percentages of the total cells. The flow cytometry results also provided simultaneous data as to the number of cells present in the pre-G1 peak, allowing determination of the number of apoptotic cells present in the population.

Levels of the proapoptotic cytosolic protein Bax were determined using confocal immunomicroscopy. Endothelial cells were grown on coverslips and exposed to glucose-specific medium for 72 h. The cells were then fixed with 4% paraformaldehyde, washed in PBS, and blocked for 90 min in 20% horse serum in 0.1 M PBS. The coverslips were incubated overnight with rabbit anti-human Bax (DAKO) at a dilution of 1:400 at 4°C and then repeatedly washed with PBS, incubated with 1:200 FITC-labeled anti-rabbit IgG (DAKO) for 90 min, and finally mounted on slides using Glycergel (DAKO). The cells were visualized with a ×63 oil-immersion objective by using an Optiscan 5009e confocal unit (Optiscan, Victoria, Australia) mounted on an Olympus BX60 microscope. Average pixel intensities within each cell were quantified using the NIH Image software.

Cellular Necrosis

Trypan blue exclusion and lactate dehydrogenase (LDH) release were both measured as markers of cellular necrosis. Cells were grown on 96-well plates and exposed to glucose-specific medium for 72 h. The cells were trypsinized, and 50-μl aliquots of a 0.1% trypan blue solution (Searle Diagnostics) in PBS were added to the cell suspensions. After 10 min, the cells were counted with a hemocytometer, and the number of cells stained with trypan blue was expressed as a percentage of the total cell number. Levels of LDH activity were determined in supernatants collected from cells exposed to the glucose-specific medium for 72 h. The spectrophotometric assay was performed by Pacific Laboratory Medicine Services (Sydney, Australia).

TGF-β Neutralization

To neutralize endogenously produced TGF-β1, a panspecific anti-human TGF-β1 antibody was added at a concentration of 30 μg/ml to cells exposed to high glucose and to the osmotic control for 72 h. This concentration is known to achieve maximum neutralization of TGF-β activity (14). Cell number, protein content, and progression through the cell cycle were measured as described above, and levels of apoptosis were assessed using DAPI stain.

Statistical Analysis

The experiments were performed at least in triplicate on a minimum of four and a maximum of seven different endothelial cell isolates. Results are expressed as a percentage of the
control value (5 mM d-glucose) except the flow cytometry data, which represent the percentage distribution of the cells within the cell cycle. Results are expressed as means ± SE. Statistical comparisons between groups were made by analysis of variance with pairwise multiple comparisons made by Fisher’s protected least significant differences test. Analyses were performed using StatView (version 5.0; Abacus Concepts, Berkley, CA). P values <0.05 were considered significant.

RESULTS

TGF-β1 Production

Under control conditions, the total amount of TGF-β1 present in the culture supernatant after 72 h was 241.0 ± 26.0 fg/cell. Exposure to high glucose for 72 h increased the total TGF-β1 release to 404.9 ± 36.4 fg/cell (P < 0.0001), corresponding to a 168.0 ± 9.0% increase compared with the control levels (Fig. 1A). Exposure to the osmotic control increased TGF-β1 production to 342.7 ± 22.9 fg/cell (142.2 ± 6.7% compared with control; P < 0.0001; Fig. 1A). This increase was significantly less than that observed with high glucose (P < 0.05).

TGF-β1 Bioactivity

Exposure of endothelial cells to high glucose for 72 h resulted in an increase in the level of βig-h3 protein expression per cell to 155.7 ± 5.7% compared with control levels (P < 0.0001). Exposure to the osmotic control resulted in an increase to 143.1 ± 7.2% compared with the control (P < 0.0001; Fig. 1B), a level that was not significantly different from the increase in activity after exposure to high glucose.

Growth Studies

Endothelial cells grown under control conditions showed a typical proliferative response, with a linear increase in cell number at 72 h indicating a doubling time of ~54 h (Fig. 2A). The initial effect of exposure to high glucose at 24 h was a significant increase in cell number compared with control levels (P < 0.001). However, continued exposure to high glucose arrested the proliferative response, resulting in a reduction in cell number at 72 h (P < 0.001; Fig. 2A). In contrast, exposure to the osmotic control resulted in a uniform slowing of the proliferative response over the 72-h period (Fig. 2A).

The total protein content per cell under control conditions reflected the characteristic trend of proliferating cells. At the 48-h time point, there was a significant increase in cellular protein content that reflected the
premitotic increase in cell mass. However, by 72 h, the protein content per cell had returned to baseline levels (Fig. 2B). In contrast, after 72 h of exposure to high glucose, there was a significant increase in the protein content per cell ($P < 0.001$; Fig. 2B). The effect of the osmotic control was markedly different from that of high glucose, with an initial significant increase in protein content at 24 h, an effect that gradually returned toward control conditions over the subsequent 48 h (Fig. 2B).

To confirm that the growth and TGF-β1 response of high glucose exposure to endothelial cells occurred under conditions that were clinically relevant and independent of hyperosmolarity, we repeated the growth studies and TGF-β1 ELISA using 15 mM glucose. After exposure to 25 mM glucose for 72 h, cell number was $73.5 \pm 2.4\%$ ($P < 0.0001$), cell protein content was $152.6 \pm 5.5\%$ ($P < 0.0001$), and total TGF-β1 release was $141.5 \pm 5.6\%$ ($P < 0.001$) compared with control values. Similarly, exposure to 15 mM glucose for 72 h reduced cell number to $76.3 \pm 2.2\%$ ($P < 0.0001$), increased cell protein to $149.5 \pm 6.9\%$ ($P < 0.0001$), and increased TGF-β1 release to $138.4 \pm 6.2\%$ ($P < 0.001$) compared with control levels (Fig. 3, A–C). Because the growth and TGF-β1 release responses were consistent between 15 and 25 mM glucose, these data provide further confirmatory evidence that hyperosmolarity is not a significant factor in the effects observed.

**Cell Cycle Analysis**

Fluorescence-activated cell sorter (FACS) analysis was used to determine the effects of high glucose and the osmotic control on the progression of endothelial cells through the cell cycle after 72 h. A typical FACS result of cells grown under control conditions is shown in Fig. 4A. The distribution of endothelial cells throughout the phases of the cell cycle after exposure to control conditions was $63.0 \pm 8.0\%$ for the G1 phase, $8.3 \pm 1.0\%$ for the S phase, and $24.5 \pm 3.1\%$ for the G2M phase. After exposure to high glucose, no significant differences were noted in the distribution of cells in either the G1 or G2M phases. However, there was a significant increase in the number of cells in the S phase ($P < 0.005$; Fig. 4B). In contrast, treatment of the cells with the osmotic control had no effect on their distribution throughout the cell cycle (Fig. 4B).

An important finding was the identification of a subpopulation of cells that were undergoing endoreduplication (defined as those polyploid cells that failed to enter mitosis but proceeded directly from a gap phase to a phase of DNA synthesis) (Fig. 5A). Under control conditions, $3.7 \pm 1.1\%$ of cells were endoreduplicative, but after exposure to high glucose for 72 h, this population increased to $6.8 \pm 0.2\%$ ($P < 0.001$), representing an $186.5 \pm 4.3\%$ increase compared with control levels (Fig. 4). In contrast, exposure to the osmotic control did not significantly alter the numbers of cells undergoing endoreduplication (Fig. 5B).

Cell cycle analysis was also performed on endothelial cells grown in 5 mM glucose treated with TGF-β1 (1

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**Fig. 3.** A: cell number after 72 h of exposure to control or to 15 or 25 mM glucose. **P < 0.01 compared with 5 mM glucose. B: cell protein content after 72 h of exposure to control or to 15 or 25 mM glucose. **P < 0.01 compared with 5 mM glucose. C: levels of total TGF-β1 released by cells after 72 h of exposure to control or to 15 or 25 mM glucose. **P < 0.01 compared with 5 mM glucose. Values are means ± SE (n = 4).
ng/ml) for 72 h to determine whether the effects of high glucose exposure could be mimicked by TGF-β1. Exposure to TGF-β1 led to an increase in the number of cells in the S phase (P < 0.05) compared with control levels (Fig. 6A), an effect that was similar to that observed after high glucose exposure. However, unlike high glucose, TGF-β1 had no effect on the levels of endoreduplication cells (Fig. 6B).

**Cyclin-Dependent Kinases and Inhibitors**

*p27^Kip1*. *p27^Kip1* is a TGF-β-inducible 27-kDa protein that inhibits the cyclin E/Cdk2 complex that controls S phase progression, and an increase in the level of this protein is associated with delayed progression through the S phase (40). High glucose induced a significant increase in *p27^Kip1* expression per cell to 162.2 ± 12.8% (n = 32; P < 0.0001) of control values. Mannitol had no significant effect on cellular *p27^Kip1* expression (118.3 ± 8.3%; n = 32) compared with control levels.

*Fig. 7B.*

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A: a representative fluorescence-activated cell sorter (FACS) analysis after 72 h of exposure. The hatched area represents the number of cells in the S phase for 5 mM glucose (left), 25 mM glucose (middle), and 20 mM mannitol (right). B: the percentage of cells in the S phase after exposure to experimental conditions for 72 h. **P < 0.01. Values are means ± SE (n = 4).

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**Fig. 7A:**

A: a representative FACS analysis after 72 h of exposure. Area 1 represents the pre-G1 apoptotic region, and area 2 represents the region of endoreduplication in 5 mM glucose (top), 25 mM glucose (middle), and 20 mM mannitol (bottom). B: the percentage of cells undergoing endoreduplication after exposure to experimental conditions for 72 h. **P < 0.01. Values are means ± SE (n = 4).
cytosolic staining with occasional puncta (Fig. 9A). In cells exposed to high glucose there was an increase in the fluorescent intensity compared with control levels and a pronounced redistribution of the Bax within the cells, suggestive of mitochondrial translocation (Fig. 9B). In contrast, cells grown in the osmotic control showed no changes in distribution or intensity of expression of Bax (Fig. 9C).

Necrosis

In the presence of high glucose, there was no significant change in the LDH release into the medium from control values. However, in cells exposed to the osmotic control, there was an increase in amount of LDH released ($P < 0.001$; Fig. 10A). This is in keeping with the findings of Wu et al. (51).

Trypan blue staining of control cells showed that 6.2 ± 0.2% of the cells were nonviable. In the presence of high glucose, this value increased to 14.6 ± 0.8% ($P < 0.05$). However, exposure to the osmotic control resulted in a further increase to 27.7 ± 3.8% ($P < 0.0001$), an effect that was significantly greater than exposure to high glucose alone ($P < 0.005$) (Fig. 10B).

Effects of Neutralization of TGF-β1 on Endothelial Cell Growth

Having demonstrated that exposure of endothelial cells to high glucose was associated with a marked reduction in cell number and increased cellular protein content at 72 h, we proceeded to measure these parameters in endothelial cells exposed to TGF-β1. Treatment of the cells with TGF-β1 (1 ng/ml) under control conditions for 72 h resulted in a reduction in cell number ($P < 0.0001$; Fig. 11A) and an increase in protein content per cell ($P < 0.0001$; Fig. 11B) compared with control levels, an effect that was similar to that observed in response to high glucose.

We then exposed the cells to high glucose in the presence of TGF-β neutralizing antibody to determine the extent to which the effects of high glucose were mediated by TGF-β. The bioactivity of the neutralizing antibody was confirmed by treating the cells with high glucose and rabbit IgG (R&D Systems), and no difference in either growth parameter was observed compared with high glucose alone (Fig. 11A and B). However, the addition of the panspecific TGF-β antibody to cells exposed to high glucose over 72 h significantly reversed the reduction in endothelial cell number seen with high glucose back to control levels (Fig. 11A). Similarly, the increase in cell protein content observed after 72 h of exposure to high glucose was abolished in the presence of panspecific TGF-β antibody (Fig. 11B). The addition of panspecific TGF-β antibody did not reverse the growth effects observed with the osmotic control (Fig. 11, A and B). These data indicated that the growth effects observed after exposure of endothelial cells to high glucose were mediated primarily through TGF-β dependent pathways, and this was a response independent of hyperosmolarity.

Fig. 6. A: the percentage of cells in the S phase after exposure to experimental conditions including TGF-β1 exposure over a 72-h period. B: the percentage of cells undergoing endoreduplication under similar conditions. *$P < 0.05$. Values are means ± SE ($n = 4$).

Apoptosis

DAPI staining of cells grown under control conditions revealed that 3.1 ± 0.4% of the cells were apoptotic after 72 h (Fig. 8). In cells exposed to high glucose there was a 152.1 ± 12.4% increase in apoptotic cells ($P < 0.05$). In contrast, there was no effect on the levels of apoptosis after 72 h of exposure to the osmotic control.

Similarly, FACS analysis revealed that under control conditions, 0.58 ± 0.6% ($n = 13$) of cells were in the pre-G1 peak that represents apoptotic cells (Fig. 5). After exposure to high glucose for 72 h, this value increased by 141.3 ± 8.5% ($P < 0.001$). No change was observed in cells exposed to the osmotic control for 72 h.

Confocal analysis of cells grown under control conditions labeled with anti-Bax antibodies showed diffuse
Effects of Neutralization of TGF-β1 on Apoptosis

We then investigated whether autocrine TGF-β1 could account for the effects of high glucose on endothelial cell apoptosis. Cells were exposed to TGF-β1 (1 ng/ml) for 72 h, and DAPI staining revealed a doubling of the number of apoptotic cells to 211.6 ± 8.1% (P < 0.0001) of those observed in control experiments. In parallel experiments, exposure to high glucose led to a lesser increase in apoptosis to 148.2 ± 10.2%. Importantly, cotreatment of the cells with TGF-β1 and high glucose resulted in an additive increase in apoptosis to 257.8 ± 7.8% (P < 0.0001). These data suggested that other additional pathways independent of TGF-β1 mediated the increased apoptosis observed with high glucose. This hypothesis was confirmed in experiments where treatment of cells with high glucose and panspecific anti-TGF-β antibody failed to reverse the increase in apoptosis observed with high glucose compared with control (Fig. 12).

DISCUSSION

The current study highlights the pathogenic effects of exposure of high glucose on the endothelial cell and defines the role of autocrine TGF-β1 in the alterations observed in cell growth, cell cycle progression, and the levels of apoptosis and cellular necrosis. In addition, it confirms that these effects are largely glucose specific and independent of any potential hyperosmolar component of elevated glucose. Furthermore, we have identified novel cell cycle effects of high glucose that are independent of the autocrine action of TGF-β1. Our results demonstrate that short-term exposure of endothelial cells to high glucose produces a hypertrophic and antiproliferative growth response, leading to a reduction in cell number with a concomitant increase in the levels of apoptosis and a subpopulation of cells undergoing endoreduplication. These findings are in keeping with the pathology observed in vivo in diabetic models (17, 24, 28, 44).

We observed an increase in the total amount of TGF-β1 released per cell as well as increased TGF-β1 bioactivity, which was confirmed in our studies by the increased expression of the TGF-β1-inducible protein βig-h3. This increase in levels of total TGF-β1 released per cell is consistent with the increased total TGF-β1 release and increased TGF-β1 mRNA expression described by others (34). The only other study to investigate the effects of high glucose on TGF-β1 bioactivity in HUVECs used the mink lung epithelial cells proliferation assay, and no increase in TGF-β1 activity was observed (5). Although the reasons for this discrepancy between the two studies remains unclear, our results are supported by the finding of increased expression of βig-h3 in atherosclerotic vessels, suggesting that TGF-β1 plays a significant role in the pathogenesis of this condition of which diabetes mellitus is a major risk factor (33). This study also showed that the increase in TGF-β1 bioactivity observed with high glucose may be partly a consequence of its increase in hypertonicity. We are the first to demonstrate that this minor in-
crease in osmolarity significantly altered endothelial cell TGF-β1 bioactivity that potentially could induce secondary effects either locally or systemically. However, because panspecific TGF-β antibody did not reverse the growth effects associated with mannitol exposure, it would appear that this local increase in TGF-β1 was insufficient in itself to mediate the endo-

Fig. 8. 4,6-Diamidino-2-phenylindole dihydrochloride hydrate (DAPI) staining of endothelial cells after 72 h of exposure to 5 mM glucose (A) and 25 mM glucose (B). Apoptotic features of chromatin condensation are indicated with arrowheads. Values are means ± SE (n = 4).

Fig. 9. Confocal images of Bax expression in cells exposed to 5 mM glucose (A), 25 mM glucose (B), and 20 mM mannitol (C) over 72 h. Values are means ± SE (n = 4).
The antiproliferative and hypertrophic effects of high glucose are reversed by the anti-TGF-β1 antibody, confirming that these effects are mediated by autocrine TGF-β1 production. These findings differ from those of Cagliero et al. (5), although in the latter studies the effects of exogenous TGF-β1 were not reversed with the blocking antibody. We observed a reduction in endothelial cell number despite an increased number of cells synthesizing DNA. These data are consistent with high glucose inducing a delay in S phase progression,

Our results demonstrate that exogenous TGF-β1 mimics the antiproliferative and hypertrophic effects of high glucose on endothelial cells in association with an increase in the number of cells in the S phase of the cell cycle. The antiproliferative and hypertrophic effects of high glucose are reversed by the anti-TGF-β1 antibody, confirming that these effects are mediated by autocrine TGF-β1 production. These findings differ from those of Cagliero et al. (5), although in the latter studies the effects of exogenous TGF-β1 were not reversed with the blocking antibody. We observed a reduction in endothelial cell number despite an increased number of cells synthesizing DNA. These data are consistent with high glucose inducing a delay in S phase progression,

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(28) and in patients, where acute hyperglycemia is not associated with sequelae of increased levels of endothelial cell apoptosis such as thrombosis. The etiology of the proapoptotic effect of high glucose on the endothelial cell is unresolved. High glucose exposure is associated with increased endothelial cell production of reactive oxygen species (ROS), and oxidative stress has been the mechanism most often implicated in the increase in the associated endothelial cell apoptosis (6, 9, 37, 51). The increased ROS appears to act through the MAPKs with downstream upregulation of caspase-3 (32). However, TGF-β1 has been demonstrated to increase ROS production in endothelial cells (35), and therefore, because TGF-β1 is proapoptotic in endothelial cells (45), we postulated that autocrine TGF-β1 mediated the apoptotic effects of high glucose. Our results demonstrated that both high glucose and TGF-β1 caused a significant increase in the levels of apoptosis and that combined treatment with TGF-β1 and high glucose had an additive effect on the levels of apoptosis. Neutralization of TGF-β did not reverse the increased apoptosis induced by high glucose, suggesting that the increased ROS associated with high glucose-induced apoptosis occurs independently of TGF-β1 levels. Although the proapoptotic effect of high glucose has been previously described (2), this is the first demonstration that high glucose-induced endothelial apoptosis is independent of TGF-β1. The finding of distinct apoptotic mechanisms is significant because patients with diabetes have elevations in both serum glucose and active TGF-β1 (29) that may, therefore, result in a deleterious additive effect on the levels of apoptosis in vivo.

Furthermore, we present evidence that the osmotic control had no effect on the levels of apoptosis, demonstrating that the proapoptotic effects of glucose were independent of hyperosmolarity. This is in keeping with previous studies, where high glucose-induced endothelial cell apoptosis was not reproduced by exposure to mannitol (51). In contrast to high glucose, in this study the osmotic control resulted in a significant increase in cell death via necrosis. In particular, because the reduction in cell number that occurred with mannitol exposure occurred independently of the cell cycle, necrosis more than apoptosis accounted for the cell numbers observed. Previously, increased ROS production has been demonstrated to occur with HUVEC exposure to high glucose but not with exposure to mannitol (51). Wu et al. (51) have also demonstrated that whereas taurine reversed the apoptosis associated with high glucose exposure, the necrosis induced by mannitol was not reversed by the antioxidant taurine. This finding confirms that high glucose induces subtle changes in local cytokine/ROS production independently of the increase in hyperosmolarity and that these subtle changes may account for the differing growth and apoptotic effects observed between these two conditions.

In summary, we have demonstrated that high glucose, TGF-β1, and osmotic stress all have independent but partially overlapping effects on the growth of endothelial cells. The changes in cell growth during exposure to high glucose occur as a result of interactions between the autocrine effects of TGF-β1 and the metabolic and hyperosmotic effects of glucose. Importantly, this is the first demonstration that some of the deleterious effects of high glucose may be mediated by increases in the populations of cells undergoing apoptosis and endoreduplication, phenomena that occur independently of TGF-β1 and hyperosmolarity.

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