Abstract—Oxygen free radicals are believed to play a key role in cellular proliferation, and increased concentrations of these molecules have been implicated in the pathogenesis of endothelial dysfunction in diabetes mellitus. Our aim was to study the role of superoxide anions in endothelial cell proliferation under conditions of normoglycemia and hyperglycemia. Human aortic endothelial cells (HAECs) and human umbilical vein endothelial cells (HUVECs) exposed to adenoviral vectors encoding CuZnSOD (AdCuZnSOD), β-galactosidase (Adβgal), or diluent (control) were cultured in normal glucose (NG, 5.5 mmol/L) or high glucose (HG, 28 mmol/L) medium. Cell proliferation was compared by use of [3H]thymidine incorporation and cell count in transduced and control cells in the setting of NG and HG. Transgene expression was detected in transduced cells by X-gal staining and by Western analysis and SOD activity assay in AdCuZnSOD-transduced cells. Superoxide production was significantly (P < 0.05) decreased in AdCuZnSOD-transduced cells cultured in both NG and HG medium. In NG, AdCuZnSOD-transduced endothelial cells had decreased proliferation compared with control cells. After 48 hours in HG, superoxide levels were increased and DNA synthesis was decreased (P < 0.05) in control and Adβgal-transduced but were not affected in AdCuZnSOD-transduced cells. In addition, after 7 days in HG, cell counts were reduced (P < 0.05) in control (73 ± 2.5%) and Adβgal-transduced (75 ± 3.4%) but not in AdCuZnSOD-transduced cells (89 ± 3.4%). These results suggest that either a deficiency or an excess of superoxide anions inhibits endothelial cell proliferation, and the inhibitory effect of increased superoxide due to hyperglycemia can be reversed by CuZnSOD overexpression. (Arterioscler Thromb Vasc Biol. 2001;21:195-200.)

Key Words: gene transfer • adenovirus • endothelium • diabetes mellitus • superoxide dismutase

Although oxygen-derived free radicals have been implicated in causing cell damage and cell death, it has become clear in recent years that they can also play a physiological role in the intracellular signaling pathways. In particular, the importance of superoxide anion as a mediator involved in the regulation of cell growth in vascular smooth muscle cells (VSMCs) has been outlined by several reports. At variance with VSMCs, sporadic data are available on redox-regulated changes in endothelial cell proliferation, because antioxidants and agonists that upregulate superoxide production have been shown to produce few or no effects on endothelial cell growth. Therefore, it has been hypothesized that endothelial cells are less susceptible to growth regulation by redox state, although some data contradict this view.

It is well established that diabetes mellitus is associated with increased oxidative stress, which is believed to play a key role in the pathogenesis of diabetic vascular dysfunction. Endothelial cells from both microvessels and macrovessels cultured in high glucose show delayed replication, abnormal cell cycling, and increased apoptosis, along with an increased expression and activity of endogenous antioxidant enzymes. It has been hypothesized that the upregulation of antioxidant enzymes by glucose is insufficient to reverse the deleterious effects of the increased oxidative stress characteristic of this condition. Glucose-induced endothelial cell toxicity may be reversed by exposure of cells to antioxidants or their precursors, whereas increased apoptosis is prevented by the administration of exogenous SOD. Taken together, these data indicate that adequate free radical scavenging is imperative for normal endothelial function and survival in diabetes mellitus.

Therapeutic approaches designed to deliver genes encoding antioxidant enzymes to intracellular sites at sustained levels and in a durable manner may have advantages over delivery of the protein in diabetic vascular disease. Disadvantages of delivery of recombinant antioxidant proteins include the short half-life of these substances in the bloodstream along with an inability to detoxify intracellular reactive oxygen species. These limitations could possibly be overcome by use of gene therapy to create an endogenous source of enzyme to provide sustained antioxidant protection. Superoxide dismutase (SOD) is responsible for scavenging O2·− in eukaryotic cells. Three isoforms of the enzyme exist, which differ in their subcellular localization as well as in the cofactors required for catalytic activity. The cytosolic isoform requires copper and zinc (CuZn), whereas the mitochondrial

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isoform requires manganese. An immunodistinct tetrameric extracellular SOD is also CuZn-dependent. Gene transfer strategies may allow the effects of various isoforms to be compared and may provide insights into the cellular source of excess free radical generation associated with hyperglycemia.

**Methods**

**Cell Culture**

Human aortic endothelial cells (HAECs) and human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics. They were cultured in modified MCDB-131 medium (EBM, Clonetics) supplemented with 2% FBS, gentamicin (50 μg/mL), bovine brain extract (12 μg/mL), hydrocortisone (1 μg/mL), and human epidermal growth factor (10 ng/mL). HAECs were first grown to confluence at 37°C in a humidified atmosphere containing 5% CO₂. Cultures between the third and the seventh subpassages were used for the experiments.

**Construction, Propagation, and Purification of Adenoviral Vectors**

A recombinant adenovirus containing the cDNA encoding the human CuZnSOD gene driven by a cytomegalovirus promoter (AdCuZnSOD) was generated as previously described. It was propagated, isolated, and quantified by standard techniques.

AdCMVLacZ (Adβgal), used in all experiments as a control, was a kind gift from Dr James M. Wilson (University of Pennsylvania, Philadelphia). Viral stocks were stored at -70°C.

**Gene Transfer to Endothelial Cells**

HAECs and HUVECs were plated at the optimal density for each experiment and cultured overnight in regular medium. For all the experiments, cells were transduced with adenoviral vectors 24 hours after plating. Cells were incubated with various multiplicities of infection (MOIs) (25 or 50) of AdCuZnSOD or Adβgal in PBS/0.5% albumin for 1 hour at 37°C. Additional cells (control) were exposed to diluent alone. The viral solution was then removed and replaced with regular techniques.

**Assessment of Transgene Expression**

Transgene expression was demonstrated by X-gal staining of Adβgal-transduced cells. Western blot analysis of CuZnSOD protein, and SOD activity assay.

**Detection of β-Galactosidase**

For X-gal staining, HAECs were transduced with increasing MOIs (0, 25, 50) of Adβgal as described above. After gene transfer, incubation was continued in normal glucose or high glucose medium for an additional 48 hours to allow transgene expression. Thereafter, cells were washed with PBS and fixed for 5 minutes in 4% paraformaldehyde, 0.4% glutaraldehyde in PBS. One milliliter of a solution containing 500 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Boehringer Mannheim Corp) was added to each experimental well and then incubated for 2 hours at 37°C. Each well was examined under a light microscope, and the efficiency of gene transfer to the endothelial monolayer was visually assessed.

**Western Blot Analysis for Human CuZnSOD Protein**

For Western blot analysis of human CuZnSOD protein, HAECs were plated at a density of 2x10⁵ in 100-mm plates, transduced the next day with adenoviral vectors at MOI 50, and incubated for 48 hours in regular medium. Then soluble proteins were extracted by lysing the pellets. After centrifugation, the supernatant was collected and total protein concentration determined by the bicinchoninic acid assay. Prestained protein markers (Bio-Rad) and 15 μg of protein were loaded on 4% stacking/15% separating SDS-PAGE. The resolved proteins were transferred to 0.2-μm nitrocellulose membrane on a semidry electrophoretic transfer system (Bio-Rad) for Western blot analysis. Blots were blocked with 5% nonfat dry milk in PBS buffer/0.1% Tween 20 overnight at 4°C. The membrane was then incubated with a sheep anti-human CuZn-SOD (1:100, Biodesign) in blocking buffer overnight at 4°C. The blots were next incubated with peroxidase-conjugated anti-sheep secondary antibody (1:2500, Biodesign) for 1 hour at room temperature. Specific CuZnSOD protein was detected by enhanced chemiluminescence (ECL, Amersham Life Science).

**Determination of SOD Activity**

HAECs were plated, transduced the next day with AdCuZnSOD or Adβgal at MOI 50, and incubated for 48 hours in medium containing 28 mmol/L glucose. Then cells were scraped with a rubber policeman and sonicated in 1× PBS/0.1% Triton X-100 (pH 7.4) on ice with two 30-second bursts. SOD activity was measured by the reduction of cytochrome c, as described. Briefly, xanthine/xanthine oxidase was used to generate O₂⁻, which was detected by the reduction of cytochrome c. Spectrophotometric measurement of the rate of reduction of cytochrome c in the presence of increasing amounts of SOD protein was performed. Total SOD activity was determined from the amount of inhibition of cytochrome c reduction.

**Measurement of Superoxide Production**

After transduction with adenoviral vectors encoding Adβgal or AdCuZnSOD, cells were grown for 48 hours in 5.5 or 28 mmol/L glucose medium. Secretion of superoxide by the endothelial cells was determined by SOD-inhibitable reduction of cytochrome c. Cells were incubated for 1 hour in phenol red–free medium in the presence of 20 μmol/L cytochrome c. O₂⁻ release was calculated from the difference of absorbance at 550 nm; a molar extinction of 21 000 was used.

**Assessment of Cell Proliferation**

Cell proliferation was determined by [H]thymidine incorporation and cell count. For both experiments, HAECs were plated at subconfluence and transduced with 50 MOI of AdCuZnSOD or Adβgal. To assess [H]thymidine incorporation, cells were first rendered quiescent for 24 hours with medium supplemented with 0.1% FBS and then stimulated for 44 hours with regular medium containing either 5.5 or 28 mmol/L glucose. [H]thymidine incorporation was determined by addition of 1 μCi of H-labeled thymidine (Amersham Life Science) for 4 hours at 37°C. Then cells were washed, DNA was extracted with 0.5N NaOH, and radioactivity was counted by scintillation spectrometry.

Cell count was performed in both HAECs and HUVECs. For this experiment, after gene transfer, cells were incubated for 7 days in regular medium containing 5.5 or 28 mmol/L glucose, which changed every 48 hours.

Cells were counted in a Coulter Counter (model ZM, Coulter Electronics Ltd.) on day 7.

**Statistical Analyses**

Differences between mean values of multiple groups were analyzed by 1-way ANOVA with Fisher analysis. Values of P≤0.05 were considered to be statistically significant.

**Results**

**Assessment of Transgene Expression in Endothelial Cells**

**Assessment of β-Galactosidase Expression Under Normal and High-Glucose Conditions**

As assessed by X-gal staining in Adβgal-transduced HAECs cultured under normal-glucose conditions, gene transfer resulted in efficient transgene expression (Figure 1). In contrast, there was no evidence of X-gal staining in control cells (Figure 1).

To assess whether incubation in high-glucose conditions might affect efficiency of transduction, transgene expression was determined after incubation in normal- or high-glucose medium for 48 hours. X-gal–positive cells were 47.2±2% in medium containing 5.5 mmol/L glucose and 51.8±2.6% in medium containing 28 mmol/L glucose (P=0.17).
Western Blot Analysis of Recombinant CuZnSOD

Western blot analysis of CuZnSOD protein showed a single 16-kDa band corresponding to human CuZnSOD in lysates prepared from AdCuZnSOD-transduced HAECs (Figure 2).

Determination of SOD Activity

Cell lysates were next examined for antioxidant enzyme activities. After transduction with AdCuZnSOD and incubation in medium containing 28 mmol/L glucose for 48 hours, total SOD activity was significantly \((P<0.0005)\) increased in AdCuZnSOD-transduced HAECs (435.8 ± 101.4 U/mg protein) compared with control (68.3 ± 8.6 U/mg protein) and Adβgal-transduced (57.6 ± 9.4 U/mg protein) cells.

Measurement of Superoxide Production

In medium containing 5.5 mmol/L glucose, \(O_2^{•−}\) production was significantly \((P<0.007)\) decreased in AdCuZnSOD-transduced HAECs (1.6 ± 0.2 nmol · h⁻¹ · well⁻¹) compared with control and Adβgal-transduced cells (2.5 ± 0.13 and 2.4 ± 0.2 nmol · h⁻¹ · well⁻¹, respectively) (Figure 3A).

After 48 hours in medium containing 28 mmol/L glucose, control and Adβgal-transduced HAECs released in the medium 4.0 ± 0.1 and 4.2 ± 0.1 nmol \(O_2^{•−}\) per hour per well, respectively (Figure 3B). Transduction with AdCuZnSOD significantly \((P<0.03)\) reduced the amount of \(O_2^{•−}\) measured in the culture medium (3.2 ± 0.2 nmol · h⁻¹ · well⁻¹) (Figure 3B).

Effect of Adenovirus-Mediated Gene Transfer on Endothelial Cell Proliferation

Under normal-glucose conditions, transduction with Adβgal resulted in a significant inhibition of DNA synthesis (Adβgal, 1347 ± 93.5 and control, 1875 ± 132.9 dpm/well, \(P<0.05\)) (Figure 4A) and cell count (Adβgal, 68.6 ± 6.4 × 10⁳ and control, 81.3 ± 9.4 × 10⁳ cells/well, \(P<0.05\)) (Figure 4B). Thus, adenoviral vector per se had an inhibitory effect on endothelial cell proliferation in this cell line. To determine whether the effect of adenovirus on cell proliferation is cell line–specific, additional experiments were performed on another endothelial cell line (HUVECs). Transduction with Adβgal did not significantly affect cell count in this endothelial cell line (Adβgal, 50.0 ± 5.0 × 10³ and control, 57.1 ± 2.4 × 10³ cells/well).

Figure 1. X-Gal staining of HAECs transduced with adoviral solutions containing increasing concentrations of Adβgal. A, 0 MOI (no virus); B, 25 MOI; C, 50 MOI.

Figure 2. Effects of CuZnSOD gene transfer on expression of CuZnSOD protein. HAECs were incubated with 50 MOI of AdCuZnSOD or Adβgal in regular medium, and incubation was continued for 48 hours more to allow expression. Medium was then removed, and cells were harvested and sonicated. Human CuZnSOD protein in cell lysate was detected by Western blot analysis. Cell lysates (15 μg protein) were loaded. CuZnSOD protein expression (lane 3) was markedly increased in AdCuZnSOD-transduced cells compared with control (lane 1) and Adβgal-transduced (lane 2) cells.

Figure 3. Effects of CuZnSOD gene transfer on \(O_2^{•−}\) production. HAECs were transduced with 50 MOI of AdCuZnSOD or Adβgal and exposed to 5.5 or 28 mmol/L glucose medium. After 48 hours, cells were washed and incubated in 1 mL serum-free, phenol red–free medium containing 20 μmol/L cytochrome c for 1 hour. \(O_2^{•−}\) levels were calculated from amount of cytochrome c reduction at 550 nm. A, \(O_2^{•−}\) production in 5.5 mmol/L glucose; B, \(O_2^{•−}\) production in 28 mmol/L glucose. Each bar represents mean±SEM of 2 values from 3 separate experiments. \(*P<0.03\) vs control and Adβgal.
**Effect of CuZnSOD Overexpression on Endothelial Cell Proliferation in Normal Glucose**

To assess the effect of superoxide anion on endothelial cell proliferation under normoglycemic conditions, adenovirus-mediated gene transfer of CuZnSOD to HAECs was performed. In normal glucose, [3H]thymidine incorporation was inhibited in AdCuZnSOD-transduced (875 ± 657.7 dpm/well) compared with Adβgal-transduced (1347 ± 93.5 dpm/well, \( P < 0.05 \)) HAECs (Figure 4A). This finding was confirmed by cell count (49.1 ± 6.5 x 10^3 cells/well in the AdCuZnSOD versus 68.6 ± 6.4 x 10^3 cells/well in the Adβgal group, \( P < 0.05 \), Figure 4B).

Transduction with AdCuZnSOD also resulted in a significant inhibition of cell proliferation in HUVECs (30.0 ± 2.4 x 10^3 cells/well versus 57.1 ± 2.4 x 10^3 in control and 50.0 ± 5.5 x 10^3 in Adβgal, \( P < 0.05 \)), confirming the role of superoxide anions in endothelial cell proliferation under conditions of normal glucose.

**Effects of High Glucose Concentrations on Cell Proliferation**

High glucose resulted in decreased proliferation in both HAECs and HUVECs. After 48 hours’ exposure to high glucose, [3H]thymidine incorporation was 86.9 ± 3.6% and 82.1 ± 2.9% of each control in normal glucose in control and Adβgal-transduced HAECs (Figure 5A), consistent with results previously reported. After 7 days in 28 mmol/L glucose, cell counts in both control and Adβgal-transduced HAECs were significantly \( (P < 0.05) \) less than the corresponding group grown in physiological glucose (73 ± 2.5% and 75 ± 3.4%, respectively) (Figure 5B). Similar cell count was obtained with HUVECs (control, 81.5 ± 5.2%; Adβgal, 63.6 ± 7.6%, \( P < 0.05 \) versus each control group in 5.5 mmol/L glucose).

**Effects of CuZnSOD Gene Transfer on Cell Proliferation in High Glucose**

The effect of CuZnSOD overexpression on endothelial cell proliferation in the setting of high glucose was next determined. In contrast to control and Adβgal-transduced cells, DNA synthesis as assessed by [3H]thymidine incorporation was not decreased in AdCuZnSOD-transduced HAECs exposed to high concentrations of glucose (Figure 5A). Furthermore, cell counts were not significantly reduced in AdCuZnSOD-transduced HAECs (Figure 5B) grown in high glucose. Similar results were showed with HUVECs (AdCuZnSOD-transduced cells, 91.3 ± 4% of the control group in 5.5 mmol/L glucose).

**Discussion**

Superoxide anions play a crucial role in cell proliferation. In the present study, we show that overexpression of CuZnSOD in endothelial cells cultured in normal glucose decreases the...
production of superoxide anion and inhibits cell proliferation. Furthermore, culturing cells in high glucose increases the production of superoxide anion and also inhibits cell proliferation. Finally, we demonstrate that the effect of high glucose on cell proliferation can be prevented by CuZnSOD overexpression. These results suggest that regulation of superoxide anions plays a crucial role in the modulation of endothelial cell proliferation and that perturbations induced by pathological states such as hyperglycemia may be overcome by gene transfer of antioxidant enzymes.

Extensive data from the literature support the role of superoxide anion as a growth-promoting factor in VSMCs. The data on the effect of superoxide on endothelial cell proliferation are sporadic and somewhat contradictory, however, because exposure to antioxidants does not affect endothelial cell growth, whereas superoxide anion appears to be involved in the regulation of basal, nonstimulated endothelial cell proliferation.

To assess the effect of SOD overexpression on cell proliferation in normal- and high-glucose conditions, we used a strategy of adenovirus-mediated gene transfer. It should be noted that adenovirus-mediated gene transfer per se inhibited proliferation of the HAEC line used in these experiments. When cells were cultured in normal glucose, thymidine incorporation and cell counts were decreased in the Adβgal group compared with control cells, suggesting a toxic effect of the vector. Adenovirus-mediated inhibition of cell proliferation has previously been described for a number of cell lines but never to our knowledge for a vascular cell line. When experiments were repeated in HUVECs, however, cell counts were not significantly decreased in Adβgal-transduced cells, suggesting that the effect may be cell line–specific. This concept is in keeping with previous results from our group using porcine coronary artery SMCs and HUVECs in which a toxic effect of an adenoviral vector encoding β-galactosidase was not observed. Thus, the sensitivity to adenovirus-mediated toxicity may vary depending on the cell line under consideration. We chose to continue our studies in high glucose with HAECs because of their strategic location in the arterial bed and because this cell line has some distinctive characteristics that are not shared by HUVECs.

When cultured in normal-glucose medium, cells expressing CuZnSOD had decreased superoxide production and agonist-induced proliferation compared with Adβgal-expressing cells and cells exposed to diluent alone. Thus, in both endothelial cell lines, overexpression of CuZnSOD was associated with decreased cell proliferation in normal glucose concentrations. Our results are similar to those observed when fibroblasts are treated with the antioxidant N-acetylcysteine, which results in inhibition of cell proliferation and suggest that superoxide anion plays a critical role in cell proliferation. Although the mechanism of this effect is unclear, a role for superoxide in the Ras signaling pathway has been suggested. These results suggest that scavenging superoxide anions by SOD overexpression in normal-glucose conditions inhibits endothelial cell proliferation and that a critical concentration of superoxide anions appears to be necessary for cell proliferation.

In the present study, we evaluated the effect of high glucose on endothelial cell proliferation. In vitro, high glucose has been demonstrated to delay endothelial cell replication, cause cell death, and trigger apoptosis via increased superoxide production. The latter effect is prevented by antioxidants. Ho et al. recently showed that reactive oxygen species induced by high glucose mediate apoptosis in endothelial cells via JNK activation, which triggers caspase 3, whereas Pieper et al. demonstrated that incubation of endothelial cells in high glucose results in the activation of nuclear factor-κB. Thus, increased apoptosis may represent one of the possible mechanisms by which increased concentrations of superoxide anion found in association with high glucose affect cell viability.

For the reasons outlined above, the data concerning the effect of high glucose concentrations on HAEC cell proliferation are expressed in relation to cell counts in normal glucose for each experimental condition. In keeping with previous reports on glucose-mediated cytotoxicity, these results clearly demonstrate that proliferation of control and Adβgal-transduced endothelial cells is inhibited by high glucose concentrations via increased generation of superoxide radicals. In the present study, culture of endothelial cells in high glucose resulted in increased superoxide generation, which was reversed by CuZnSOD overexpression. This effect was associated with prevention of glucose-mediated decrease of cell proliferation, thus suggesting that increased superoxide generation was responsible for the inhibition of endothelial cell proliferation.

It was demonstrated previously that delayed endothelial cell replication due to oxidative stress in the setting of high glucose may be reversed by administration of SOD protein. However, this approach is limited by the fact that the exogenously administered SOD remains in an extracellular location, whereas superoxide radical is generated inside the cell. In contrast, overexpression of CuZnSOD via gene transfer has previously been shown to result in the correct cytoplasmic location of the transgene product. In addition, this approach may result in a longer duration of protein expression.

In conclusion, these data suggest that either a deficiency or an excess of superoxide levels inhibits endothelial cell proliferation. Furthermore, the inhibitory effect of increased superoxide anion levels on endothelial cell proliferation observed in diabetes mellitus may be reversed by overexpression of superoxide dismutase.

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References


Superoxide Anions and Endothelial Cell Proliferation in Normoglycemia and Hyperglycemia
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