Glucose-6-phosphate dehydrogenase (G6PD), the first enzyme of the pentose phosphate pathway, is the principal intracellular source of NADPH. NADPH is utilized as a cofactor by vascular endothelial cell nitric-oxide synthase (eNOS) to generate nitric oxide (NO). To determine whether G6PD modulates NO-mediated angiogenesis, we decreased G6PD expression in bovine aortic endothelial cells using an antisense oligodeoxynucleotide to G6PD or increased G6PD expression by adenoviral gene transfer, and we examined vascular endothelial growth factor (VEGF)-stimulated endothelial cell proliferation, migration, and capillary-like tube formation. Deficient G6PD activity was associated with a significant decrease in endothelial cell proliferation, migration, and tube formation, whereas increased G6PD activity promoted these processes. VEGF-stimulated eNOS activity and NO production were decreased significantly in endothelial cells with deficient G6PD activity and enhanced in G6PD-overexpressing cells. In addition, G6PD-deficient cells demonstrated decreased tyrosine phosphorylation of the VEGF receptor Flk-1/KDR, Akt, and eNOS compared with cells with normal G6PD activity, whereas overexpression of G6PD enhanced phosphorylation of Flk-1/KDR, Akt, and eNOS. In the Pretsch mouse, a murine model of G6PD deficiency, vascular endothelial growth factor (VEGF)-stimulated angiogenesis and vascular permeability are significantly attenuated (6).

Glucose-6-phosphate dehydrogenase (G6PD), the first and rate-limiting enzyme in the pentose phosphate pathway, is the principal intracellular source of NADPH. NADPH, in turn, is utilized directly as a cofactor for eNOS and, indirectly, to maintain levels of another important cofactor, tetrahydrobiopterin, via de novo synthesis and the dihydrofolate reductase salvage pathway. In this manner, G6PD regulates eNOS activity and NO levels. In this study, we demonstrate that G6PD activity modulates endothelial cell migration, proliferation, and tube formation by mediating NO levels. G6PD may, therefore, serve as a novel regulatory determinant of the angiogenic phenotype.

Experimental Procedures

Antisense Transfection and Recombinant Adenovirus Gene Transfer—Bovine aortic endothelial cells (BAEC) (Cell Systems Co, Kirkland, WA) were grown to confluence in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. To decrease G6PD expression, an antisense phosphorothioate oligodeoxynucleotide to G6PD mRNA (AS) or a scrambled sequence control (SS) was utilized as described previously (7). To increase G6PD expression, a recombinant adenovirus encoding rat G6PD cDNA under control of the cytomegalovirus promoter, AdG6PD, or an empty viral vector, Ad, were utilized as described previously (8). G6PD protein expression was determined by Western blotting (Fig. 1A), and corresponding NADPH levels were measured as described previously (7, 8) (Fig. 12). Experiments were conducted on cells from passages 4–10.

Thymidine Incorporation Assay—BAEC (1 × 10⁴) were seeded in triplicate 24-well plates, placed in reduced serum (1.0%) media for 24 h, and stimulated with vascular endothelial growth factor (VEGF) (100 ng/ml) in 1.0% serum for 12 h in the presence of 1 μCi/ml [methyl-³H]-thymidine. After 12 h, cells were washed with PBS and fixed in 10% trichloroacetic acid at 4 °C for 16 h, washed with 100% ethanol, incubated with 1 N NaOH

Glucose-6-phosphate dehydrogenase (G6PD) modulates vascular endothelial growth factor-mediated angiogenesis.

Received for publication, February 5, 2003, and in revised form, May 15, 2003

Published, JBC Papers in Press, May 30, 2003, DOI 10.1074/jbc.M301293200

Jane A. Leopold‡, Jennifer Walker‡, Anne W. Scribner‡, Barbara Voetsch‡, Ying-Yi Zhang‡, Alexander J. Loscalzo‡, Robert C. Stanton‡, and Joseph Loscalzo‡

From the ‡Whitaker Cardiovascular Institute and Evans Department of Medicine, Boston University School of Medicine, Boston, Massachusetts 02118 and ¶Joslin Diabetes Center, Harvard Medical School, Boston, Massachusetts 02215

Angiogenesis, the formation of new blood vessels in response to tissue ischemia or injury, is dependent upon a coordinated sequence of events involving vascular endothelial cell migration, proliferation, and tube formation (1, 2). Initially, vascular endothelial cells must acquire an angiogenic phenotype to migrate toward an angiogenic stimulus, proliferate behind the front of migration, and differentiate to form endothelial tubes and capillary-like structures.

Nitric oxide (NO) has been shown to modulate angiogenesis by mediating growth factor-stimulated endothelial cell migration and proliferation. Nitric oxide is permissive for endothelial cell migration and enhances directional migration by inducing a switch from a stationary to a mobile phenotype (3, 4). In addition, NO promotes endothelial cell proliferation, and proliferating endothelial cells demonstrate increased expression of the endothelial isoform of nitric-oxide synthase (eNOS) compared with quiescent cells (5). In vivo studies utilizing eNOS−/− mice have demonstrated the absolute requirement for endothelium-derived NO for effective angiogenesis. In this murine model, compared with mice with normal eNOS activity, vascular endothelial growth factor (VEGF)-stimulated angiogenesis and vascular permeability are significantly attenuated (6).

The abbreviations used are: NO, nitric oxide; G6PD, glucose-6-phosphate dehydrogenase; eNOS, endothelial cell nitric-oxide synthase; VEGF, vascular endothelial growth factor; BAEC, Bovine aortic endothelial cells; AS, antisense; SS, scrambled sequence; PBS, phosphate-buffered saline; l-NAME, l-N-nitroarginine methyl ester; l-NMMA, l-N-nitroarginine monomethyl ester; hpf, high powered field; ROS, reactive oxygen species; WT, wild type; HEMI, hemizygous.

* This work was supported in part by National Institutes of Health Grants F90 HL5993, HL58976, HL61795, and HL04399. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Whitaker Cardiovascular Institute, Boston University School of Medicine, 700 Albany St., CABR W-507, Boston, MA 02118. Tel.: 617-638-4893; Fax: 617-638-4066; E-mail: Jane.Leopold@bmc.org.
for 30 min at 37 °C, and neutralized with 10 N HCl. Thymidine incorpo-
ration was measured by liquid scintillation counting.

Cell Migration—Migration was assessed by a cell wounding assay in
BAEC grown to confluence in a P100 dish and synchronized in 1%
serum for 24 h. A longitudinal incision was made in the midline of
the plate with a sterile scalpel, and cells were scraped from one-half of
the plate and stimulated with VEGF (100 ng/ml) for 12 h. After this time,
cells that migrated across the midline were visualized using a Nikon TE
300 microscope, and 5 images per high powered field (hpf) per plate
were captured digitally. Cells that crossed the midline were counted
and averaged per plate. Data are presented as migration index.

Tube Formation—In vitro formation of capillary-like tube structures
was examined using Matrigel. Matrigel (0.5 ml) was polymerized on
dual-chamber microscope slides. Cells were then plated on Matrigel
in full-growth media for 1 h. Once the cells were seeded, the media were
replaced with media containing 1% serum or VEGF (100 ng/ml). Tube
formation was visualized using an inverted microscope (Nikon TE 300)
equipped with digital imaging. For each treatment, 10 high power field
images were captured, and area of tubes/networks formed was quanti-
fied using Scion Corp. (NIH Image) area analysis with background sub-
traction and averaged. Data are presented as density units.

eNOS Activity—eNOS activity was measured in intact cells without
the addition of exogenous cofactors as described previously (9).

Western Blotting—Western blot analyses were performed as de-
scribed previously (9). For analysis, equal amounts of cellular proteins
(50 μg/lane) were resolved by SDS-PAGE and transferred to polyvinyl-
dene difluoride membrane. After blocking in 5% milk solution, mem-
branes were probed with anti-phospho-tyrosine (clone 4G10) (Upstate
Biotechnology, Inc.), anti-Flk-1/KDR (Santa Cruz Biotechnology), anti-
endothelial nitric oxide synthase (eNOS) antibodies (Cell Signaling Tech-
ologies) overnight at 4 °C. G6PD protein expression was determined by Western blotting. B, corresponding NAPDH levels were
determined. Data are presented as mean ± S.E., n = 4, * p < 0.001 versus SS-EC; ** p < 0.001 versus AD-EC.

RESULTS

**G6PD and NADPH levels**

A. BAEC were transfected with an antisense oligodeoxynucleotide to G6PD mRNA to decrease G6PD expression (AS-EC), or a scrambled control (SS-EC), or infected with an adenovirus encoding rat G6PD cDNA (AdG6PD-EC) to increase G6PD expression. eNOS protein expression (A) was determined by Western blotting. B, corresponding NADPH levels were determined. Data are presented as mean ± S.E., n = 4, * p < 0.001 versus SS-EC; ** p < 0.001 versus AD-EC.

In Vivo Matrigel Plug Assay and Cell Recovery—Matrigel (0.5 ml)
was injected subcutaneously in the ventral groin area. One side was
injected with Matrigel alone and the other with Matrigel mixed with
VEGF (100 ng/ml). In this manner, each animal serves as its own
control. After 14 days, the mice were euthanized by CO2 inhala-
tion. Matrigel plugs were excised and fixed in 10% formalin, subjected to
an ethanol dehydration series, and embedded in paraffin. Serial sections
(10 μm) were cut using a cryotome and applied to glass slides. Slides
were deparaffinized and stained with hematoxylin and eosin. To re-
cover cells, the excised Matrigel plugs were minced with a sterile
scalpel, passed 10 times through a 14-gauge needle, treated with BD™
Cell Recovery Solution for 1 h at 4 °C, centrifuged, and subjected to
immunoprecipitation as described above to assay for proteins of
interest.

Immunohistochemistry—Immunohistochemistry on deparaffinized
slides was performed using a rabbit polyclonal anti-von Willebrand
factor antibody (Santa Cruz Biotechnology, Inc.) at 1:50, as described
previously (14). An angiogenic response was quantified by cell counts
from 10 high power fields per section and image analysis using Scion
Corp. (NIH Image) with background subtraction to determine the area
occupied by endothelial cells.

Statistical Analysis—Continuous data were expressed as mean ±
S.E. Comparison between groups was performed by Student’s paired
two-tailed t test. Two-way analysis of variance was used to examine
differences in response to treatments between groups, with post hoc
an analysis performed by the method of Student-Newman-Keuls. A
p value of <0.05 was considered significant.

RESULTS

**G6PD and Endothelial Cell Proliferation**—To examine the
role of G6PD in VEGF-mediated endothelial cell proliferation,
BAEC were transfected with an antisense oligodeoxynucleotide to
G6PD mRNA with a 36% transfection efficiency to decrease
G6PD expression and activity by 76% (AS-EC) or a scrambled
control sequence (SS-EC), and cells were stimulated with
VEGF (100 ng/ml) for 12 h. Compared with SS-EC, AS-EC
demonstrated a significant decrease in basal and VEGF-stim-
ulated [3H]thymidine incorporation (Fig. 2A). The observed
decrease in cell proliferation was not the result of increased cell
death as determined by lactate dehydrogenase activity in the
media (data not shown).

To determine the contribution of NO to VEGF-stimulated
endothelial cell proliferation, SS-EC and AS-EC were pre-treated for 1 h with t-NAME (1 mmol/liter), to inhibit both NO' and superoxide production by eNOS, or t-NMMA (100 μmol/liter), to inhibit NO' generation by eNOS, and stimulated with VEGF in the presence of these inhibitors. In SS-EC stimulated with VEGF, $[^3H]$thymidine incorporation was decreased significantly in cells treated with t-NAME (53,874 ± 3,848 versus 41,932 ± 5,330 cpm, p < 0.01) or t-NMMA (53,874 ± 3,848 versus 44,187 ± 3,267 cpm, p < 0.01). Similarly, AS-EC stimulated with VEGF demonstrated a decrease in $[^3H]$thymidine incorporation in the presence of t-NAME (32,156 ± 4,561 versus 26,897 ± 2,142 cpm, p < 0.05) and t-NMMA (32,156 ± 4,561 versus 26,116 ± 4,668 cpm, p < 0.05).

We next sought to determine whether overexpression of G6PD would augment cell proliferation. To perform these studies, BAEC were infected with AdG6PD (AdG6PD-EC) at a multiplicity of infection = 10 plaque-forming units/cell with a 90–95% infection efficiency to increase G6PD activity 5-fold.

Fig. 2. G6PD and endothelial cell proliferation. Cell proliferation was measured following stimulation with VEGF (100 ng/ml) for 12 h in a $[^3H]$thymidine incorporation assay in BAEC transfected with an anti-sense oligodeoxynucleotide to G6PD mRNA to decrease G6PD expression (AS-EC), or a scrambled control (SS-EC) (A), and BAEC infected with an adenovirus encoding rat G6PD cDNA (AdG6PD-EC) to increase G6PD expression, or an empty viral vector (Ad-EC) (B). Data are presented as mean ± S.E., n = 6, *p < 0.01 versus SS-EC, Ad-EC; **p, p < 0.001 versus SS-EC, Ad-EC.

As G6PD overexpression was associated with enhanced endothelial cell migration, we performed the in vitro Matrigel assay. SS-EC and AS-EC were plated on Matrigel and stimulated with VEGF for 12 h. Under basal conditions, SS-EC plated on Matrigel formed tubes and networks, and the area occupied by SS-EC endothelial tubes was increased significantly following exposure to VEGF (9,911 ± 640 versus 24,729 ± 3,311 units, p < 0.001). Similarly, AS-EC formed endothelial tubes and the area occupied by these networks was not significantly different from that observed with SS-EC (9,911 ± 640 versus 8,424 ± 1,005 units, p = not significant); however, when stimulated with VEGF, AS-EC tube formation was decreased significantly compared with SS-EC (10,554 ± 895 versus 24,729 ± 3,311 units, p < 0.002) (Fig. 4A).

As G6PD overexpression was associated with enhanced endothelial cell migration and proliferation, it is not surprising that compared with Ad-EC, tube formation was significantly increased in AdG6PD-EC under basal conditions (19,549 ± 1,286 versus 23,6 ± 7.0 units, p < 0.01) as did t-NMMA (51.9 ± 4.2 versus 21.6 ± 7.3 units, p < 0.003). Interestingly, in AS-EC, VEGF did not significantly increase endothelial cell migration, and t-NAME or t-NMMA did not alter this response.

G6PD and Endothelial Cell Migration—To examine the influence of G6PD on VEGF-stimulated endothelial cell migration, we performed a cell wounding assay. SS-EC and AS-EC were incubated with VEGF, and cell migration across the midline was observed. After 12 h, VEGF markedly increased cell migration across the midline in SS-EC (44 ± 4 versus 142 ± 16 cells/hpf, p < 0.001), an effect that was not observed in AS-EC (27 ± 11 versus 35 ± 5 cells/hpf, p = not significant) (Fig. 3).

These findings were confirmed utilizing a modified Boyden chamber assay to determine whether G6PD activity influenced directed VEGF-mediated endothelial cell migration. SS-EC and AS-EC were stimulated with VEGF (100 ng/ml) for 12 h during which time cells could migrate across the membrane. In SS-EC treated with VEGF, there was a significant increase in fluorescence (13.3 ± 2.6 versus 55.3 ± 6.6 units, p < 0.001), indicating an increase in cell migration, that was abrogated in AS-EC with deficient G6PD activity (14.1 ± 4.8 versus 20.2 ± 9.9 units, p = not significant).

We next sought to determine the contribution of NO' to endothelial cell migration. By utilizing the modified Boyden chamber assay, SS-EC or AS-EC were stimulated with VEGF for 12 h in the presence or absence of L-NAME (1 mmol/liter) or L-NMMA (100 μmol/liter). In SS-EC, t-NAME significantly decreased cell migration in VEGF-stimulated cells (51.9 ± 4.2 versus 23.6 ± 7.0 units, p < 0.01) as did t-NMMA (51.9 ± 4.2 versus 21.6 ± 7.3 units, p < 0.003). Interestingly, in AS-EC, VEGF did not significantly increase endothelial cell migration, and t-NAME or t-NMMA did not alter this response.

To determine whether overexpression of G6PD would promote directed endothelial cell migration, we overexpressed G6PD in endothelial cells and examined migration in a modified Boyden chamber assay. Compared with Ad-EC, AdG6PD-EC demonstrated a significant increase in fluorescence under basal conditions (16.4 ± 3.2 versus 35.1 ± 4.7 units, p < 0.009) and following stimulation with VEGF (51.9 ± 3.1 versus 98.8 ± 7.1 units, p < 0.001). In the presence of t-NAME, fluorescence was decreased in both unstimulated AdG6PD-EC (35.1 ± 4.7 versus 21.5 ± 3.4 units, p < 0.01) and VEGF-stimulated cells (98.8 ± 10.1 versus 36.4 ± 4.3 units, p < 0.001), suggesting that increased G6PD expression mediates endothelial cell migration by increasing NO' levels.

G6PD, eNOS Activity, and NO' Levels—These observations suggest that one mechanism by which G6PD influences endothelial cell proliferation, migration, and tube formation is to regulate eNOS activity and, thereby, bioavailable NO'. To examine the effect of G6PD expression on eNOS activity, we measured eNOS activity in intact cells (without the addition of exogenous cofactors) (Fig. 5A). In AS-EC, compared with SS-EC, eNOS activity was significantly decreased (12,728 ± 488
was quantified by counting cells that migrated across the midline in 10 hpf per plate. Data are presented as mean ± S.E., n = 6. * , p < 0.001 versus no addition; **, p < 0.001 versus SS-EC.

To examine the mechanism(s) by which G6PD mediates NO’ production, we first performed immunoprecipitation studies to determine whether G6PD co-localizes with eNOS. Under basal conditions, G6PD and eNOS co-localize, and following stimulation with VEGF, co-localization is enhanced (Fig. 5C). We next sought to determine whether G6PD influences activation of the VEGF receptor Flk-1/KDR to modulate Akt-eNOS activation. Interestingly, in AS-EC, tyrosine phosphorylation of a 230-kDa band, consistent with Flk-1/KDR, was decreased at both 5 and 15 min compared with what was observed in SS-EC resulting in decreased phosphorylation of Akt and eNOS in AS-EC compared with SS-EC (Fig. 5D). In contrast, in AdG6PD-EC, tyrosine phosphorylation of the 230-kDa band was enhanced at 5 and 15 min resulting in increased phosphorylation of Akt and eNOS compared with Ad-EC (Fig. 5D).

G6PD, VEGF, and ROS Accumulation—It has been demonstrated recently that VEGF promotes angiogenesis in human umbilical vein endothelial cells by stimulating NAD(P)H oxidase to increase ROS formation (16). Therefore, we measured ROS levels by 2’7’-dichlorodihydrofluorescein diacetate fluorescence in endothelial cells with decreased or increased G6PD activity to determine whether our observations could be explained by changes in ROS levels. Interestingly, in AS-EC exposed to VEGF, ROS levels were significantly higher than in SS-EC (248 ± 27 versus 180 ± 22 units, p < 0.01), and conversely, in AdG6PD-EC, ROS levels were lower than in Ad-EC (156 ± 14 versus 197 ± 12 units, p < 0.01). These findings suggest that G6PD, which is recognized as an antioxidant enzyme in endothelial cells, may additionally modulate ROS accumulation by influencing NO’ production to mediate the redox milieu to a level favorable for endothelial cell proliferation, migration, and tube formation.

Ex Vivo Aorta Implants and Vessel Outgrowth—We next examined the effect of G6PD on VEGF-mediated angiogenesis using an in vivo model of G6PD deficiency, the Pretsch mouse. Compared with C3H background mice (WT), hemizygous male G6PD mice (HEMI) demonstrate only 31% of WT G6PD activity.

We next examined the aorta outgrowth, we explanted thoracic aortas from WT and HEMI
mice, sectioned them into rings, and embedded the rings in a collagen matrix. The rings were then observed over 6 days for vessel outgrowth. By day 3, there was significant outgrowth from the WT rings compared with the rings from the HEMI mice, and by day 6, there was a marked difference between outgrowth observed from WT and HEMI mice rings (Fig. 6). To quantify these observations, the area occupied by vessel outgrowth was determined using area image analysis with back-ground subtraction. At day 3, the area occupied by vessel outgrowth was significantly greater in aortic rings from WT compared with HEMI mice (6,037 ± 975 versus 2,767 ± 391 units, p < 0.001), and this effect was more pronounced by day 6 (10,057 ± 713 versus 3,475 ± 295 units, p < 0.001).

In Vivo Matrigel Assay—To examine the effect of G6PD activity on angiogenesis in vivo, we performed an in vivo Matrigel migration assay in WT and HEMI mice. Each mouse was injected on one side with either Matrigel alone or Matrigel supplemented with VEGF, and the plugs were examined after 14 days. In WT mice, there is noticeable cell migration into the Matrigel plug, and this response is increased in Matrigel supplemented with VEGF (12 ± 1 versus 52 ± 2 cells/hpf, p < 0.001). In contrast, in HEMI mice, there is a marked decrease in cell migration into the Matrigel plug compared with WT mice, and exposure to VEGF only modestly improved this response (6 ± 1 versus 9 ± 1 cells/hpf, p < 0.03) (Fig. 7A). These findings confirm that G6PD modulates angiogenesis in vivo.

To determine that the cells that migrated into the Matrigel were endothelial cells, the Matrigel sections were immunostained for von Willebrand factor and counted. In Matrigel sections from WT mice, 91% of cells stained positive for von Willebrand factor, whereas in HEMI mice 89% of cells stained positive for von Willebrand factor.

**Fig. 5.** G6PD, eNOS, and NO\(^{\bullet}\) production. eNOS activity and NO\(^{\bullet}\) production were measured in BAEC transfected with an antisense oligodeoxynucleotide to G6PD mRNA (AS-EC) or a scrambled control (SS-EC), or infected with an empty adenoviral vector (Ad-EC) or an adenoviral vector encoding G6PD cDNA (AdG6PD-EC). A, eNOS activity was measured in intact cells stimulated with VEGF (100 ng/ml) by the conversion of L-[\(^{3}\)H]arginine to L-[\(^{3}\)H]citrulline in the absence of exogenous cofactors. Results are measured as counts per min, and data are expressed as mean ± S.E. from three experiments performed in duplicate. *, p < 0.001 versus VEGF; #, p < 0.004 versus SS-EC; **, p < 0.001 versus Ad-EC; ##, p < 0.001 versus Ad-EC. B, NO\(^{\bullet}\) production was measured in real time with a nitric oxide probe in cells stimulated with VEGF (100 ng/ml). NO\(^{\bullet}\) production was measured as nmoles/liter, and the area under the curve was integrated and expressed in units. Data are expressed as mean ± S.E. from three experiments. *, p < 0.008 versus SS-EC; **, p < 0.001 versus Ad-EC. C, co-localization of G6PD and eNOS was examined in BAEC stimulated with VEGF and immunoprecipitated with an antibody to eNOS or G6PD and immunoblotted with an antibody to eNOS or G6PD. D, the influence of G6PD on VEGF-mediated activation of eNOS was determined by examining phosphorylation of a 230-kDa protein corresponding to the VEGF receptor Flk-1/KDR, Akt, and eNOS by Western blotting in BAEC transfected with an antisense oligodeoxynucleotide to G6PD mRNA (AS-EC) or a scrambled control (SS-EC) (left), or infected with an empty adenoviral vector (Ad-EC) or an adenoviral vector encoding G6PD cDNA (AdG6PD-EC) (right). Blots are representative of three experiments.

**Fig. 6.** Ex vivo aortic rings and vessel outgrowth. Thoracic aortas were harvested from C3H wild-type (WT) and G6PD-deficient X\(^{\text{Yhi}}\) hemizygous (HEMI) mice. Aortas were dissected free of adventitia, sectioned into 1-mm rings, and embedded in a collagen matrix. Rings were examined at high power magnification (×40) after 3 days (A and B) and 6 days (C and D) for vessel outgrowth.

Willebrand factor and counted. In Matrigel sections from WT mice, 91% of cells stained positive for von Willebrand factor, whereas in HEMI mice 89% of cells stained positive for von Willebrand factor.
in plugs supplemented with AdG6PD, there was a marked increase in cell migration into the plug, which was enhanced in plugs treated with VEGF (15 ± 1 versus 48 ± 4 cells/hpf, p < 0.001). These findings demonstrate that the WT phenotype may be restored successfully in G6PD-deficient mice by local gene transfer of G6PD.

We next recovered cells from Ad- and AdG6PD-treated Matrigel plugs that had been implanted for 7 or 14 days, and we examined these cells for G6PD expression. Interestingly, G6PD expression was increased in cells from AdG6PD-treated plugs at 7 days compared with Ad-treated plugs, and this effect remained present at 14 days, although somewhat diminished (Fig. 7C). To confirm that our in vitro observations were operative in vivo, we next examined recovered cells for phosphorylation of Flk-1/KDR, Akt, and eNOS. In cells recovered from AdG6PD-treated plugs, there was enhanced phosphorylation of a 230-kDa protein consistent with Flk-1/KDR at day 7 resulting in increased phosphorylation of Akt and eNOS compared with cells recovered from Ad-treated plugs. This effect was also present, although to a lesser extent, at day 14 (Fig. 7C).

**DISCUSSION**

In these studies, we found that G6PD significantly influenced VEGF-mediated vascular endothelial cell proliferation, migration, and tube formation in an in vitro cell culture model and an in vivo murine model. Decreased G6PD expression and activity were associated with an impaired response to VEGF-stimulated cell proliferation, migration, and formation of tubes and networks in a Matrigel matrix. In contrast, increased G6PD expression and activity enhanced these processes. G6PD modulated these events, in part, by influencing basal and VEGF-stimulated eNOS activity and NO levels. We have demonstrated that this may occur as a result of G6PD co-localizing with eNOS. In addition, we also demonstrate that G6PD influences tyrosine phosphorylation of the VEGF receptor Flk-1/KDR and, in turn, phosphorylation of Akt and eNOS. In endothelial cells with deficient G6PD activity, we have demonstrated previously that eNOS may “uncouple” to generate superoxide in preference to NO (7). To determine that a decrease in eNOS-mediated NO production, and not an increase in superoxide generation by eNOS, accounted for the diminished response to VEGF in G6PD-deficient cells, it was therefore critical to utilize the eNOS inhibitors lNAME, which inhibits superoxide and NO generation by eNOS, and l-NMMA, which inhibits only NO production by eNOS (17).

In studies performed in the presence of these inhibitors, we demonstrated that eNOS-mediated NO generation was the critical determinant of basal and VEGF-stimulated endothelial cell proliferation, migration, and tube formation. These findings were confirmed by direct measurement of eNOS activity and NO levels.

Importantly, by utilizing a murine model of G6PD deficiency, we demonstrated that G6PD regulates endothelial cell proliferation, migration, and tube formation in vivo. In aortas harvested from G6PD-deficient mice, there is decreased vessel outgrowth in a collagen matrix preparation compared with aortas from animals with normal G6PD activity. Similarly, in an in vivo Matrigel assay, there was a diminished endothelial cell response under basal conditions and following stimulation with VEGF in G6PD-deficient mice. Interestingly, the G6PD-deficient phenotype could be “rescued” by adding adenovirus encoding G6PD to the Matrigel plug, presumably resulting in local gene transfer of G6PD, to restore endothelial cell migration and proliferation to the degree observed in wild-type mice with normal levels of G6PD activity. The validity of this approach has been confirmed recently in a murine model using a different cDNA (18). By analyzing cells recovered from the plugs, we demonstrate further that there is increased expres-
tion of G6PD in these cells. Furthermore, this results in increased tyrosine phosphorylation of Flik-1/KDR, Akt, and eNOS in vivo to confirm our in vitro findings.

The association between G6PD activity and cell proliferation has been well described in several non-vascular cell types (19, 20). Growth factors, such as platelet-derived growth factor and epidermal growth factor, have been shown to increase cell proliferation by increasing both basal G6PD activity and G6PD expression (19). Deficient G6PD activity is associated with decreased cell proliferation in both steroid receptor-positive and receptor-negative breast cancer cell lines (20), COS-7 cells, Swiss 3T3, Balb/c 3T3, and A21 fibroblasts (19). In contrast, increased G6PD expression enhances cell proliferation. Adenoviral gene transfer of G6PD to increase G6PD expression 2—3-fold in COS-7 cells resulted in a marked increase in [3H]thymidine incorporation (19). Similarly, NIH 3T3 cells transfected with human G6PD cDNA exhibited contact- and anchorage-independent growth (21). Taken together, these findings demonstrate that G6PD activity is associated with cell proliferation in non-vascular cell lines. We now provide evidence that G6PD activity is associated with vascular endothelial cell proliferation.

To date, there have been no studies to evaluate the influence of G6PD on cell migration. In fact, the current evidence linking G6PD to cell migration is indirect; G6PD activity has been associated with cell motility, as a surrogate for migration, in spermatogenic cells. In a rat model, treatment with agents that decreased G6PD activity resulted in a reduction in sperm motility (22). In our studies, we provide the first evidence that G6PD importantly modulates directed vascular endothelial cell migration.

The mechanism(s) and/or effectors by which G6PD enhances acquisition of an angiogenic phenotype remain incompletely characterized; however, it has been demonstrated that intra-cellular NADPH levels, and not the production of riboses by G6PD, are the critical determinant of cell growth (19). This suggests that one mechanism by which G6PD regulates endothelial cell migration, proliferation, and tube formation is by synthesizing NO via eNOS, which has an absolute requirement for NADPH as a cofactor.

The relationship between G6PD and NO activity has been demonstrated in several cell lines. For example, NO production is impaired in G6PD-deficient granulocytes stimulated with lipopolysaccharide or phorbol 12-myristate 13-acetate. Interestingly, this response resulted from a decrease in activity, not expression, of the inducible form of NOS (23). Similarly, in peritoneal macrophages from p53−/− mice treated with dehydroepiandrosterone (at concentrations that inhibit G6PD activity), NO activity and indices of NO production were significantly decreased (24).

In vascular endothelial cells, we have shown previously that G6PD influences eNOS activity by regulating substrate availability. G6PD-deficient endothelial cells produce decreased levels of bioavailable NO in response to agonists because of eNOS “uncoupling” to generate reactive oxygen species in preference to NO (7). This uncoupling of eNOS has been shown to occur when cofactors, such as NADPH and/or tetrahydrobiopterin, which is synthesized and salvaged in NADPH-dependent reactions, are depleted. Furthermore, gene transfer of G6PD to increase G6PD expression and activity in aortic endothelial cells resulted in enhanced NADPH levels, which in turn increased eNOS activity and NO generation measured as cGMP, nitrate, and nitrite levels (10).

There are additional mechanisms by which G6PD may influence eNOS activity. Co-localization of G6PD with eNOS represents one mechanism by which G6PD may modulate NO production. In fact, it has been shown previously that G6PD and nitric-oxide synthase co-localize in brush cells of rat stomach and pancreas (25). We now demonstrate in vascular endothelial cells that G6PD co-localizes with eNOS. In addition, VEGF-stimulated NO production has been shown to occur via phosphorylation of the VEGF receptor Flik-1/KDR to phosphorylate and activate Akt and thereby eNOS (26, 27). Activation of this signaling pathway has been implicated in angiogenesis both in vitro and in vivo (6, 28). It is likely, but not determined that decreased phosphorylation of Flik-1/KDR, Akt, and eNOS associated with deficient G6PD activity results in diminished endothelial proliferation, migration, and tube formation both in vitro and in vivo and that increased G6PD activity enhances these responses.

These studies provide novel insight into the role of G6PD in vascular endothelial cell proliferation, migration, and tube formation. In vascular endothelial cells, G6PD activity correlates with the response to agonist-mediated cell proliferation and migration, which, in turn, determines tube formation. This effect occurs in part by the influence of G6PD on eNOS activity to generate NO. Therefore, G6PD, by modulating eNOS activity to regulate vascular endothelial cell proliferation, migration, and tube formation, is a critical regulatory determinant of the angiogenic phenotype.

Acknowledgments—We thank Dr. Shane Thomas for helpful suggestions and Dr. Diane Handy and Antoniette Hayes for their assistance.

REFERENCES

1. Carmeliet, P. (2000) Nat. Med. 6, 389–395
2. Vaillie, S., Vittet, D., and Peig, J. J. (2001) Lab. Invest. 81, 439–452
3. Ziche, M., Morbidelli, L., Masini, E., Amerini, S., Granger, H. J., Maggi, C. A., Geggotti, P., and Ledda, F. (1994) J. Clin. Invest. 94, 2036–2044
4. Noori, E., Paresleni, T., Srivastava, N., Weber, P., Bahou, W. F., Peunova, N., and Goligorsky, M. S. (1996) Am. J. Physiol. 270, C794–C802
5. Zöllner, S., Auberle, S., Harvey, S. R., Polkoff, M. A., and Rubanyi, G. M. (2000) Endothelium 7, 169–184
6. Fukumura, D., Gohngi, T., Tsuchida, A., Irimi, Y., Ang, J., Yun, C. O., Buerk, D. G., Huang, P. L., and Jain, R. K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 2604–2609
7. Leopold, J. A., Cap, A., Sribner, A. H., Stanton, R. C., and Loscalzo, J. (2001) FASEB J. 15, 1771–1773
8. Zhang, Y. Y., Walker, J. J., Huang, A., Keaney, J. F., Clish, C. B., Serhan, C. N., and Loscalzo, J. (2002) Biochem. J. 361, 267–276
9. Uittenbogaard, A., Shaul, P. W., Yuhanna, I. S., Blair, A., and Smart, E. J. (2000) J. Biol. Chem. 275, 11278–11283
10. Leopold, J. A., Zhang, Y. Y., Sribner, A. H., Stanton, R. C., and Loscalzo, J. (2003) Arterioscler. Thromb. Vasc. Biol. 23, 411–417
11. Burbridge, M. F., and West, D. C. (2001) in Angiogenesis Protocols (Murray, J. C., ed.) pp. 185–204, Humana Press, Totowa, NJ
12. Pretsch, W., Charles, D. J., and Merkle, S. (1988) Biochem. Genet. 26, 89–103
13. Sanders, S., Smith, D. P., Thomas, G. A., and Williams, E. D. (1997) Mutat. Res. 374, 79–87
14. Bierhaus, A., PTG, F., Torgerson, R., Torgerson, R., and Bierhaus, A., PTG, F. (1988) Biochem. Genet. 26, 89–103
15. Bouloumie, A., Schini-Kerth, V. B., and Russe, R. (1999) Cardiovasc. Res. 41, 773–780
16. Ushio-Fukai, M., Tang, Y., Fukai, T., Diakabol, S. I., Ma, Y., Fujimoto, M., Quinn, M. T., Pagano, P. J., Johnson, C., and Alexander, R. W. (2002) Circ. Res. 91, 1160–1167
17. Xia, Y., Tsai, A. L., Berka, V., and Zweier, J. L. (1998) J. Biol. Chem. 273, 25894–25898
18. Lucerna, M., Mechtcheriakova, D., Kadi, A., Schabauer, G., Schaefer, R., Gruber, F., Koshelnick, Y., Muller, H. D., Isbrucker, K., Claus, M., Binder, B. R., and Hofer, E. (2003) J. Biol. Chem. 278, 11433–11440
19. Tian, W. N., Braunstein, L. D., Pang, J., Stuhlmeier, K. M., Xi, Q. C., Tian, X., and Stanton, R. C. (1998) J. Biol. Chem. 273, 10609–10617
20. De Monaco, M., Pizzini, A., Gatto, V., Leonardi, L., Gallo, M., Brindisi, E., and Boccuzzi, G. (1997) Br. J. Cancer 76, 589–592
21. Kuo, W. Y., and Tang, T. K. (1998) Free Radic. Biol. Med. 24, 1130–1138
22. Pant, N., Prasad, A. K., Srivastava, S. C., Shankar, R., and Srivastava, S. P. (1995) Hum. Exp. Toxicol. 14, 889–894
23. Tsai, K. J., Hung, I. J., Chow, C. K., Stern, A., Chao, S. S., and Chiu, D. T. (1998) FEBS Lett. 436, 411–414
24. Mei, J. J., Hursting, S. D., Perkins, S. N., and Phang, J. M. (1998) Cancer Lett. 129, 191–197
25. Kugler, P., Hofer, D., Mayer, B., and Drencichka, D. (1994) J. Histochem. Cytochem. 42, 1317–1323
26. Fulton, D., Gratten, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Frank, T. F., Papapetropoulos, A., and Sessa, W. C. (1999) Nature 399, 597–611
27. Dimmeler, S., Fleming, I., Fisslthaler, B., Herrmann, C., Busse, R., and Zeiher, A. M. (1999) Nature 399, 601–605
Glucose-6-phosphate Dehydrogenase Modulates Vascular Endothelial Growth Factor-mediated Angiogenesis

Jane A. Leopold, Jennifer Walker, Anne W. Scribner, Barbara Voetsch, Ying-Yi Zhang, Alexander J. Loscalzo, Robert C. Stanton and Joseph Loscalzo

J. Biol. Chem. 2003, 278:32100-32106.
doi: 10.1074/jbc.M301293200 originally published online May 30, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M301293200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 27 references, 7 of which can be accessed free at http://www.jbc.org/content/278/34/32100.full.html#ref-list-1