NAD(P)H:quinone Oxidoreductase 1-Compromised Human Bone Marrow Endothelial Cells Exhibit Decreased Adhesion Molecule Expression and CD34⁺ Hematopoietic Cell Adhesion

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ABSTRACT

NAD(P)H:quinone oxidoreductase 1 (NQO1) deficiency resulting from a homozygous NQO1*2 polymorphism has been associated with an increased risk of benzene-induced myeloid toxicity and a variety of de novo and therapy-induced leukemias. Endothelial cells in human bone marrow form one of the two known hematopoietic stem cell microenvironments and are one of the major cell types that express NQO1 in bone marrow. We have used a transformed human bone marrow endothelial cell (TrHBMEC) line to study the potential impact of a lack of NQO1 activity on adhesion molecule [endothelial leukocyte adhesion molecule 1 (E-selectin), vascular cell adhesion molecule (VCAM)-1, and intercellular adhesion molecule (ICAM)-1] expression and functional adhesion to bone marrow progenitor cells. We used both 5-methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]indole-4,7-dione (ES936), a mechanism-based inhibitor of NQO1, and anti-NQO1 small interfering RNA to abrogate NQO1 activity. Real-time reverse transcription-polymerase chain reaction data demonstrated a significant inhibition of tumor necrosis factor (TNF)α-induced E-selectin mRNA levels after ES936 pretreatment. Immunoblot assays demonstrated a significant reduction in TNFα-stimulated E-selectin, VCAM-1, and ICAM-1 proteins after inhibition or knockdown of NQO1. The mechanisms underlying this effect remain undefined, but modulation of nuclear factor-κB (p65), c-Jun, and activating transcription factor 2, transcriptional regulators of adhesion molecules, were observed after inhibition or knockdown of NQO1. Decreased levels of E-selectin, VCAM-1, and ICAM-1 also resulted in a functional deficit in adhesion. A parallel plate flow chamber study demonstrated a marked reduction in CD34⁺ cell (KG1a) adhesion to NQO1-deficient TrHBMECs relative to controls. The reduced adhesive ability of TrHBMECs may affect the function of the vascular stem cell niche and also may contribute to the increased susceptibility of polymorphic individuals lacking NQO1 to leukemias and hematotoxicants such as benzene.

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NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase) is a flavin-containing quinone reductase (Ernster, 1967; Blanchet et al., 2004) that is polymorphic in humans (Traver et al., 1997). The NQO1*2 polymorphism is a single-nucleotide polymorphism, defined as a C-to-T substitution at position 609 of the human NQO1 cDNA, corresponding to a proline-to-serine change at position 187 of the protein (Traver et al., 1997). The mutant NQO1*2 protein is rapidly degraded by the ubiquitin proteasomal system, resulting in an absence or only trace levels of NQO1 protein in individuals carrying the NQO1*1/*2 genotype (Moran et al., 1999). Benzene is an occupational and environmental pollutant, and chronic exposure to benzene can induce aplastic anemia, myelodysplasia, and acute myeloid leukemia (Travis et al., 1994). Benzene requires metabolism to exert toxicity, and benzene-derived quinones are considered to play a major role in its toxicity (Ross, 2000). NQO1 can metabolize benzene-derived quinones and with respect to benzene-induced toxic-
ity, there is convincing evidence that NQO1 acts as a detoxi-
fication enzyme. NQO1 knockout mice demonstrated increased
benzene-induced hematotoxicity (Bauer et al., 2003), and an
increased risk of benzene poisoning associated with the
NQO1*2 polymorphism has been demonstrated in individu-
als occupationally exposed to benzene (Rothman et al., 1997).
However, increased risks of a variety of de novo and therapy-
induced leukemias also have been associated with the
NQO1*2 polymorphism ( Larson et al., 1999; Wiemels et al.,
1999; Naoe et al., 2000; Krajnovic et al., 2002; Smith et al.,
2002; Ross and Siegel, 2004), and unchallenged NQO1 knock-
out mice demonstrate myeloid hyperplasia ( Long et al.,
2002). The mechanisms whereby a lack of NQO1 due to the
NQO1*2 polymorphism predisposes to both benzene-induced
myeloid toxicity and a variety of leukemias not associated
with quinone exposure remain unclear. However, NQO1 has
multiple functions and is part of a coordinated response to
stress ( Ross and Siegel, 2004), stabilizes proteins such as p53
against proteasomal degradation (Asher et al., 2002), and can
function in an antioxidant role ( Siegel et al., 2004).
Bone marrow stroma and particularly endothelial cells are
intimately associated with developing blood cells, and up-
regulation of cell adhesion molecules in response to cytokine
induction is an important endothelial cell function ( Man-
tovani et al., 1992). Adhesion molecules expressed by endo-
thelial cells are important regulators of hematopoiesis and
contribute to stem cell/progenitor cell homing and mobiliza-
tion (Schweitzer et al., 1997; Wright et al., 2001; Avecella
et al., 2004; Kopp et al., 2005). Altered hematopoiesis was
reported in mice deficient in both P- and E-selectin (Frenette
et al., 1996). The administration of antibodies against VCAM-1
ligand VLA-4 or CD44 [ a major E-selectin ligand on hemato-
poietic progenitor cells (HPCs); Dimitroff et al., 2001] in mice
led to a significant increase in circulating stem cells ( Ver-
meulen et al., 1998). In preliminary microarray studies, we
demonstrated that inhibition of NQO1 led to decreased
VCAM-1 expression (Zhou et al., 2007). Because cytokines
and adhesion molecules play key roles in regulating hematopo-
iesis, we hypothesized that NQO1 may influence cytokine-
stimulated hematopoietic cell adhesion to bone marrow
endothelial cells via modulation of adhesion molecule expres-
sion. In this study, we have used transformed human bone marrow
endothelial cells (TrHBMECs) as a model system to investigate
the effect of a lack of NQO1 on adhesion molecule expression
and adhesion of CD34+ cells. TrHBMEC is a bone marrow
 sinusoidal endothelial cell line that has been used in studies of
the adhesion and homing of HPCs (Schweitzer et al., 1997).
TNFα stimulates adhesion molecule production, and we have
characterized adhesion molecule responses to TNFα induction
as a result of mechanism-based inhibition of NQO1 activity by
ES936 (Winski et al., 2001) or genetic knockdown of NQO1 in
TrHBMECs. We have focused on E-selectin, critical for HPC
rolling and adhering on TrHBMECs (Schweitzer et al., 1997),
and on both VCAM-1 and ICAM-1, which contribute to firm
adhesion of HPCs to TrHBMECs (Schweitzer et al., 1997). We
have also examined the functional consequences of modulation
of NQO1 activity on adhesion of CD34+ cells to TrHBMECs and
explored potential mechanisms by which NQO1 deficiency
might down-regulate adhesion molecule expression in response
to TNFα induction.

Materials and Methods

Materials. NADH, 2,6-dichlorophenol-indophenol, dicumarol,
and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide were
obtained from Sigma-Aldrich (St. Louis, MO). The mechanism-
based NQO1 inhibitor ES936 was synthesized by Prof. C. J. Moody
(School of Chemistry, University of Nottingham, Nottingham, UK)
based on published procedures (Naylor et al., 1998). RNeasy kit and
QuantiTect SYBR Green PCR kits were obtained from QIAGEN
(Va lencia, CA). NE-PER nuclear and cytoplasmic extraction re-
agents were purchased from Pierce Chemical (Rockford, IL). Trans-
fection reagent Lipofectamine 2000 was purchased from Invitrogen
( Carlsbad, CA). siRNA targeting human NQO1 and scrambled
siRNA control were both purchased from Dharmacon RNA Technol-
ologies (Lafayette, CO). Mouse anti-E-selectin primary antibodies good
for nonreducing conditions were obtained from Abcam Inc. (Cam-
bridge, MA). Mouse anti-VCAM-1 and ICAM-1 monoclonal antibod-
ies and rabbit anti-IκBα, p65, and phospho-p65 (Ser529) polyclonal
antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa
Cruz, CA). Mouse anti-phospho-IκBα (Ser32/36) monoclonal anti-ody and rabbit anti-PARP, phospho-c-Jun (Ser63), and phospho-
ATF2 (Thr71) antibodies were obtained from Cell Signaling Tech-
nology (Danvers, MA). Mouse anti-NQO1 monoclonal antibody (clone
A180) was generated in our laboratory. Mouse anti-β-actin antibody was
obtained from Sigma-Aldrich. TNFα was purchased from R&D
Systems (Minneapolis, MN). Parallel plate flow chamber was ob-
tained from Glytech Corporation (Manassas, VA). Cells were propagated in RPMI 1640
medium with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in 5% CO2. Cells
with passage number ranging from 19 to 25 were used in this study. KG1a
hematopoietic cell line was obtained from American Type Culture
Collection (Manassas, VA). Cells were propagated in RPMI 1640
medium with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in 5% CO2. KG1a
cells with passage number ranging from 3 to 7 were used in the cell-cell adhesion
study.

NQO1 Activity Assay. Cell culture medium was aspirated, and
cells were washed twice with phosphate-buffered saline and scraped
into 25 mM Tris-HCl, pH 7.4, containing 250 mM sucrose and 5 μM
FAD, followed by probe sonication on ice. Sonicates were then
centrifuged at 13,000 rpm for 10 min at 4°C, and the supernatant was
assayed for NQO1 activity and protein concentration. NQO1 activity
assays were performed as described by Ernst (1967) and modified
by Benzson et al. (1980) using 2,6-dichlorophenol-indophenol as a
substrate. Reactions (1 ml) contained 25 mM Tris-HCl, pH 7.4, 0.7
mg/ml bovine serum albumin, 0.2 mM NADH, and 40 μM 2,6-dichlo-
rophenol-indophenol. Reactions were started by the addition of a
small volume (2–5 μl) of cell supernatant. Reactions were performed in
the absence and presence of 20 μM dicumarol. NQO1 activity
is defined as the dicumarol-inhibitable reduction of 2,6-dichlorophenol-
indophenol measured at 600 nm at 25°C.

siRNA Transfection. TrHBMECs were transfected with siRNA
targeting human NQO1 or scrambled siRNA control (Dharmacon RNA
Technologies) using Lipofectamine 2000 (Invitrogen) transfection
reagent. The procedure was performed as suggested in the
manufacturer’s protocol. Knockdowns were extremely efficient as
indicated by immunoblotting (see appropriate figures), and no
knockdown of the closely related protein NQO2 was detected in
TrHBMECs.

Quantitative Real-Time RT-PCR of the E-selectin Gene. The
relative amount of E-selectin mRNA was assessed using Quanti-
tect SYBR Green Real-Time PCR kit and primer set QTO015358
(QIAGEN) with an ABI 7500 system (Applied Biosystems, Foster
β-2-Macroglobulin was used as the internal control. DMSO was used as vehicle control for ES936 treatment. All of the reactions were run in triplicate. The fold increase of E-selectin mRNA in TrHBMECs after ES936 and TNFα treatment, normalized to an endogenous β-2-macroglobulin reference and relative to DMSO and TNFα control, is given by 2^ΔΔCT, where ΔΔCT = ΔCT_{E-selectin} - ΔCT_{β-2-macroglobulin} + ΔCT_{E-selectin} + ΔCT_{β-2-macroglobulin} (Lowry et al., 1951). Protein samples were diluted in 5× Laemmli sample buffer, heated to 90°C for 5 min, and then separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes in 25 mM Tris, 192 mM glycine containing 20% (v/v) methanol at 110 V for 1 h. After transfer, membranes were placed overnight in blocking buffer (10 mM Tris-HCl, pH 8, containing 125 mM NaCl, 0.2% (v/v) Tween 20, and 5% (w/v) nonfat dry milk or 5% (w/v) bovine serum albumin). Antibody dilutions were based on manufacturer's recommendations. For all analyses, horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG were diluted at 1:5000 in blocking buffer for 0.5 to 1 h at room temperature. Protein bands were visualized using luminol-based enhanced chemiluminescence as described by the manufacturer (PerkinElmer Life and Analytical Sciences, Boston, MA). For nonreducing immunoblotting, dithiothreitol and/or β-mercaptoethanol were removed from the sample buffer; primary antibodies were used at the dilutions of 1:1000 for anti-E-selectin. Densitometry analysis software used was Photoshop 7.0 (Adobe Systems, Mountain View, CA), and experimental data were normalized to loading controls.

Flow Chamber Adhesion Assay. Under defined shear stress, adhesion was measured under flow conditions in a parallel plate flow perfusion chamber. The assay was performed as described previously (Schweitzer et al., 1997), with minor modifications. In brief, TrHBMECs were pretreated with siRNA for 72 h or ES936 for 2 h to abrogate NQO1 protein or activity, and the near confluent gelatin-coated 35-mm plates were used in the circular flow chamber system (Glycotech Corporation) at 37°C. Before adhesion experiments, TrHBMECs were stimulated with TNFα (10 ng/ml) for 4 h to induce adhesion molecule expression. CD34+ KG1a cells were used in a concentration of 0.5 million/ml resuspended in HEPES buffer (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1.2 mM KH₂PO₄, 1 mM MgSO₄, 5 mM glucose, and 1 mM CaCl₂, pH 7.4). KG1a cells were perfused through the system for 5 min at a shear stress of 6.0 dyne/cm². After cell perfusion, a relatively high-shear stress of 6.0 dyne/cm² 1-min wash was applied using HEPES buffer to detach rolling or very weakly adhering cells. Cells remaining adhered after this wash were considered firmly adhered. To quantify the firm adhesion results, typically at least 10 randomized high-power fields (total surface ≥1 mm²) in each plate were chosen to count the firmly adhered KG1a cells. Chamber corner areas were avoided, and only the portions of the plates that were covered with greater than 95% endothelial cells were evaluated. For each treatment at least three to five independent experiments were performed to determine cell-cell adhesion.

Statistical Analysis. Values are presented as the mean ± S.D. Statistical significance was evaluated using the Student’s t test for comparisons between two mean values. A value of p < 0.05 was considered significant. All experiments were performed at least three times.
decreased adhesion of CD34/H9251 ES936 followed by TNF arrows). However, pretreatment of TrHBMECs with Fig. 6, A and B (smaller adhered KG1a cells indicated by increased KG1a cell adhesion to TrHBMEC as shown in Treatment of TrHBMECs with TNF would be impaired in NQO1-compromised TrHBMECs. under (Fig. 6, B and C). Adherent cells were counted as described means of three independent experiments. n = 3, *p < 0.05.

ES936 Treatment Inhibited TNFα-Induced VCAM-1 and ICAM-1 Protein Up-Regulation in TrHBMECs. We extended our studies to test the effect of inhibition of NQO1 activity on TNFα-induced VCAM-1 and ICAM-1 expression. VCAM-1 and ICAM-1 expression reaches a peak 6 h after TNFα induction (Schweitzer et al., 1997; Fig. 4A). Immunoblot analysis of VCAM-1 and ICAM-1 protein levels demonstrated a decrease of VCAM-1 and ICAM-1 proteins in ES936-pretreated samples, particularly 6 h after TNFα stimulation (Fig. 4, A and B).

Knockdown of NQO1 Protein in TrHBMECs by Use of siRNA Also Inhibits TNFα-Induced Up-Regulation of E-selectin, VCAM-1, and ICAM-1. To further confirm the regulation of adhesion molecule expression by NQO1, anti-NQO1 siRNA was used to knockdown NQO1 protein. Seventy-two hours after siRNA transfection, TNFα was then added to induce adhesion molecule expression. Immunoblot assays demonstrated the efficient knockdown of NQO1 and marked reduction of E-selectin, VCAM-1, and ICAM-1 protein levels after TNFα stimulation as a result of NQO1 knockdown (Fig. 5, A and B).

Inhibition or Knockdown of NQO1 Resulted in Impaired Adhesion of KG1a CD34+ Hematopoietic Cells to TrHBMECs after TNFα Stimulation. To examine a functional endpoint of impaired adhesion molecule expression, we explored whether adhesion of CD34+ KG1a cells would be impaired in NQO1-compromised TrHBMECs. Treatment of TrHBMECs with TNFα alone markedly increased KG1a cell adhesion to TrHBMEC as shown in Fig. 6, A and B (smaller adhered KG1a cells indicated by arrows). However, pretreatment of TrHBMECs with ES936 followed by TNFα stimulation resulted in markedly decreased adhesion of CD34+ KG1a cells to TrHBMECs (Fig. 6, B and C). Adherent cells were counted as described under Materials and Methods. Statistical analysis (Fig. 6D) demonstrated a significant decrease in KG1a CD34+ cell adhesion to TrHBMECs as a result of inhibition of NQO1. We then extended these experiments to knockdown of NQO1 in TrHBMECs, and pretreatment with siRNA against NQO1 significantly reduced TNFα-stimulated adhesion of KG1a cells to TrHBMECs (Fig. 6E). Both inhibition and knockdown of NQO1 in TrHBMECs led to a reduction of TNFα-stimulated adhesion of approximately 60 to 70% (Fig. 6, A–E).

Inhibition or Knockdown of NQO1 Did Not Inhibit TNFα-Induced IkBα Phosphorylation and Degradation in TrHBMECs. An important regulatory pathway of TNFα-induced adhesion molecule expression is the NF-κB signaling pathway (Collins et al., 1995). Classical NF-κB activation relies on phosphorylation followed by degradation of its bound inhibitors belonging to the IκB family and translocation of free NF-κB from cytosol to the nucleus. IκBα is a well characterized inhibitor of NF-κB, and its up-regulation can significantly inhibit TNFα-induced gene expression (Kordula et al., 2000; Son et al., 2004). We therefore examined total IκBα and phosphorylated IκBα levels after TNFα stimulation in TrHBMECs. However, little difference in the kinetics of either phosphorylation of IκBα or degradation/for-
mation of IκBα could be observed in NQO1-compromised cells (Fig. 7, A and B). Levels of IκBα and its phosphorylated form were similar in anti-NQO1 siRNA-treated cells relative to scrambled siRNA-treated controls and in ES936-treated cells relative to vehicle controls (DMSO) (Fig. 7, A and B).

Inhibition or Knockdown of NQO1 Did Not Block TNFα-Induced Nuclear Translocation of NF-κB Subunit p65 in TrHBMECs. The induction of IκBα phosphorylation and degradation indicated that NQO1 deficiency did not alter the upstream events of the NF-κB signaling pathway. We then examined total nuclear p65 levels in TrHBMECs to determine whether NQO1 deficiency had any effects on p65 translocation. As shown in Fig. 8, TNFα significantly induced p65 nuclear translocation; however, little difference could be observed in NQO1-compromised cells. Levels of nuclear p65 after TNFα induction were similar in anti-NQO1 siRNA-treated cells relative to scrambled siRNA treated controls and in ES936-treated cells relative to vehicle controls (DMSO) (Fig. 8, A and B).

Inhibition or Knockdown of NQO1 Resulted in Lower Phospho-p65, c-Jun, and ATF2 Levels in TrHBMEC Nucleus in Response to TNFα Induction. Although NQO1 did not inhibit TNFα-induced nuclear translocation of NF-κB subunit p65, NQO1 deficiency could alter nuclear NF-κB activity by altering events such as p65 phosphorylation, degradation, and DNA binding. We therefore examined phospho-p65 levels in TrHBMECs (Fig. 9, A and B). Inhibition or knockdown of NQO1 led to decreased amounts of phospho-p65 in TrHBMEC nuclear fractions (as determined by the same time point pairwise comparison between the control and treatment groups normalized to the PARP loading control). Because TNFα also regulates E-selectin expression via parallel c-Jun and ATF2 pathways and because phosphorylation of both c-Jun and ATF2 contribute to TNFα-induced E-selectin promoter activity in human endothelial cells (Min and Pober, 1997; Read et al., 1997), we also examined phospho-c-Jun and ATF2 levels. NQO1-deficient TrHBMECs exhibit lower amounts of both nuclear phospho-c-Jun and ATF2 proteins in response to TNFα stimulation (Fig. 9, A and B). These findings may provide a potential mechanism by which NQO1 modulates adhesion molecule expression.
Discussion

In this work, we used a transformed human bone marrow endothelial cell line to study the consequences of a lack of NQO1 activity in this important bone marrow compartment. Our data demonstrated that inhibition of NQO1 activity led to a reduction in TNFα-stimulated E-selectin, VCAM-1, and ICAM-1 protein levels in TrHBMECs. It is important to note that decreased adhesion molecule levels also led to altered functional adhesion. Inhibition or knockdown of NQO1 and decreased adhesion molecule levels led to a marked decrease in TNFα-induced CD34+ hematopoietic cell adhesion to TrHBMECs in the order of 60 to 70%.

The main hematopoietic system resides in bone marrow and is in close contact with the stromal microenvironment, which supports hematopoietic stem cell growth and differentiation. The bone marrow stroma is composed of a variety of different cell types providing structural and functional support for hematopoiesis: endothelial cells, adipocytes, smooth muscle cells, reticular cells, osteoblasts, and stromal fibroblasts. Among these cell types, endothelial cells have a particular biological relevance. Recent advances reveal the identity of two niches that play an important role in the homeostatic regulation of HSCs: the osteoblastic niche (osteoblasts and hematopoietic cells) and the vascular niche (hematopoietic cells and endothelial cells) (Caplan and Den...
The vascular niche offers an alternative niche for mobilized stem cells that promotes proliferation and further differentiation or maturation and release into the circulatory system (Avecilla et al., 2004; Kopp et al., 2005). Because blood cells can easily penetrate sinusoids that are formed by a single layer of endothelial cells, residence of HSCs in the vascular niche enables robust and rapid response to hematopoietic stress and immediate release of HSCs into the bloodstream upon cytokine or chemokine exposure (Wright et al., 2001). Adhesion of HSCs to endothelial cells is an important part of these regulatory mechanisms (Frenette et al., 1996; Vermeulen et al., 1998; Li and Li, 2006).

In this study, we used both a mechanism-based inhibitor and siRNA to inhibit or knockdown NQO1 in TrHBMECs. NQO1-compromised TrHBMECs served as a model system to determine the effects of a lack of NQO1 activity on adhesion molecule expression in human bone marrow endothelial cells. Previous work in TrHBMECs using blocking antibodies has shown critical contributions of E-selectin to rolling and adhesion of CD34+/H11001 cells, whereas a combination of VCAM-1 and ICAM-1 antibodies markedly reduced firm adhesion of CD34+/H11001 cells to TrHBMECs (Schweitzer et al., 1997). We demonstrated that either mechanism-based inhibition or siRNA-mediated knockdown of NQO1 resulted in down-regulation of TNFα/H9251-induced E-selectin, VCAM-1, and ICAM-1 expression in TrHBMECs. Similar down-regulation of TNFα/H9251-induced E-selectin after inhibition of NQO1 activity also was

**Fig. 7.** Inhibition or knockdown of NQO1 did not inhibit TNFα/H9251-induced IkBα phosphorylation and degradation in TrHBMECs. A, TrHBMECs were transfected with siRNAs (either siRNA targeting NQO1 or scrambled siRNA control) for 72 h and then exposed to TNFα/H9251 (10 ng/ml) for the indicated times. IkBα, phospho-IkBα, and NQO1 protein levels were examined in TrHBMEC extracts by immunoblot analysis. β-Actin was included as a loading control. The blot is representative of at least three independent experiments. B, TrHBMECs were pretreated with ES936 for 2 h and then exposed to TNFα/H9251 (10 ng/ml) for the indicated times; IkBα and phospho-IkBα protein levels were examined in TrHBMEC extracts by immunoblot analysis. β-Actin was included as a loading control. The blot is representative of at least three independent experiments.

**Fig. 8.** Inhibition or knockdown of NQO1 did not block TNFα/H9251-induced nuclear translocation of NF-κB subunit p65 in TrHBMECs. A, TrHBMECs were transfected with siRNAs (either siRNA targeting NQO1 or scrambled siRNA control) for 72 h and then exposed to TNFα/H9251 (10 ng/ml) for the indicated times. Nuclear extracts were prepared, and p65 and NQO1 protein levels were examined by immunoblot analysis. PARP was included as a loading control. The blot is representative of at least three independent experiments. B, TrHBMECs were pretreated with ES936 for 2 h and then exposed to TNFα/H9251 (10 ng/ml) for the indicated times. Nuclear extracts were prepared and p65 protein level was examined by immunoblot analysis. PARP was included as a loading control. The blot is representative of at least three independent experiments.

**Fig. 9.** Inhibition or knockdown of NQO1 resulted in lower phospho-p65, c-Jun, and ATF2 levels in TrHBMEC nucleus in response to TNFα/H9251 induction. A, TrHBMECs were transfected with siRNA (either siRNA targeting NQO1 or scrambled siRNA control) for 72 h and then exposed to TNFα/H9251 (10 ng/ml) for the indicated times. Nuclear extracts were prepared, and phospho-p65, c-Jun, ATF2, and NQO1 protein levels were examined by immunoblot analysis. PARP was included as a loading control. The blot is representative of at least three independent experiments. Fold change is a same time point pairwise comparison between the control and siNQO1 treatment groups normalized to the PARP loading control. The blot is representative of at least three independent experiments. Fold change is a same time point pairwise comparison between the control and treatment groups normalized to the PARP loading control.
In human umbilical vein endothelial cells (data not shown). Furthermore, inhibition or knockdown of NQO1 was demonstrated to inhibit TNF-α-induced CD34+ hematopoietic cell adhesion to TrHBMMECs. In the hematopoietic system, many cell-surface and adhesion molecules, including E-selectin and VCAM-1, are implicated in the cross-talk between HSCs and their niches, resulting in modulation of HSC mobilization, recruitment to the vascular niche, or homing to the bone marrow (Frenette et al., 1996; Vermeulen et al., 1998; Avecella et al., 2004; Kopp et al., 2005; Li and Li, 2006). Consequently, the altered expression of E-selectin, VCAM-1, and ICAM-1 after inhibition of NQO1 catalytic activity or NQO1 knockdown and the subsequent functional deficit in adhesion of CD34+ cells to human bone marrow endothelial cells may affect proliferation and differentiation of HSCs. It is also conceivable that this impaired functioning of the vascular stem cell niche may provide a unifying hypothesis for the increased incidence of leukemias and the more pronounced toxic effects of benzene in individuals lacking NQO1 due to the presence of the homozygous NQO1*2 polymorphism. Polymorphisms in both TNFα and VCAM-1 have also been associated with increased susceptibility to benzene poisoning in occupationally exposed individuals (Lan et al., 2005; Lv et al., 2007).

Mechanisms whereby inhibition or knockdown of NQO1 might result in decreased adhesion molecule expression remain undefined, but some clues are provided by our own and previous work (Ahn et al., 2006). The NF-κB and JNK signaling pathways have been shown to be critical in TNFα-induced up-regulation of adhesion molecules (Collins et al., 1995; Min and Pober, 1997; Read et al., 1997). It is important to note that NQO1 deficiency was also reported to be able to regulate the TNFα signaling pathway in mouse keratinocytes and HCT116 cells by inhibiting IκBα phosphorylation and JNK activation (Ahn et al., 2006). Our data also suggest a role for NQO1 in the NF-κB and JNK signaling pathways, but it is interesting that we did not find altered IκBα phosphorylation and p65 translocation in TrHBMMECs as demonstrated previously in keratinocytes and in colon cancer cells (Ahn et al., 2006). However, our data demonstrate decreased nuclear levels of phospho-p65, c-Jun, and ATF2 in TrHBMMECs after inhibition or knockdown of NQO1 (Fig. 9, A and B). There is not an absolute inhibition of TNFα-stimulated transcription factor levels in the nucleus, but this is in agreement with the degree of reduction in adhesion molecule expression observed after NQO1 inhibition or knockdown (Figs. 3–5). Whether the observed reduction in nuclear levels of phospho-p65, c-Jun, and ATF2 resulted from decreased phosphorylation, enhanced degradation, or accelerated shuttling back to cytoplasm needs further investigation. Although the relative importance of each of these regulators remains unclear, the collective changes in the nuclear levels of such key transcription factors may provide a potential mechanism whereby knockdown or inhibition of NQO1 could influence adhesion molecule expression. It is interesting to note that the benzene metabolite hydroquinone also has been shown to inhibit NF-κB activation in human bone marrow progenitor cells (Kerzic et al., 2003).

In summary, our data have demonstrated that inhibition or knockdown of NQO1 abrogated TNF-α-induced E-selectin, VCAM-1, and ICAM-1 expression in TrHBMMECs, leading to reduced CD34+ hematopoietic cell adhesion. Given the documented roles of these adhesion molecules in HSC development and differentiation, it is possible that such alterations may play a role in hematopoietic pathology and the increased susceptibility of polymorphic individuals lacking NQO1 to hematotoxicants such as benzene.

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