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NAD(P)H: quinone oxidoreductase 1 expression in human bone marrow endothelial cells

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Abstract

NAD(P)H:quinone oxidoreductase 1 (NQO1) participates in the detoxification of many environmental quinones and related compounds. Recent studies have suggested that individuals with a polymorphism in NQO1 (NQO1*2), which results in a decrease (heterozygous, NQO1*1/*2) or a total loss (homozygous, NQO1*2/*2) of NQO1, may be at increased risk for the development of leukemias. Previous studies have failed to detect NQO1 in freshly aspirated bone marrow including Ficoll-purified mononuclear cells and purified $CD34^+$ hematopoietic progenitor stem cells. In these studies we examined human bone marrow core biopsies by immunohistochemistry using monoclonal antibodies directed against NQO1. These studies revealed that NQO1 was expressed in human bone marrow but expression of NQO1 was limited to bone marrow endothelium and adipocytes. To confirm the expression of NQO1 in bone marrow endothelial cells we examined an immortalized human bone marrow endothelial cell line (HBMEC-60) for NQO1 protein expression. Immunoblot analysis and an activity assay confirmed the expression of NQO1 in HBMEC-60. These data demonstrate that NQO1 is present in human bone marrow. The increased risk of leukemia associated with a deficit in NQO1 levels due to the NQO1*2 polymorphism may reflect impaired quinone detoxification and an increased susceptibility of endothelial cells in bone marrow to environmental insults.

Keywords: Benzene; Polymorphism; DT-diaphorase; NQO1; Bone marrow endothelial cells

1. Introduction

NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase, EC 1.6.99.2) is a cytosolic flavoenzyme which catalyzes the two-electron reduction of a broad range of substrates. NQO1 is classified as a detoxification enzyme primarily because of its ability to reduce quinone substrates directly to their hydroquinone derivatives bypassing the redox-cycling semiquinone.

A polymorphism in NQO1 has been characterized (NQO1*2), a C to T substitution at position 609 of the human NQO1 cDNA that codes for a proline to serine amino acid change at position

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187 of the protein (Traver et al., 1997). Genotype–phenotype studies have shown those individuals homozygous for the NQO1*2 polymorphism are deficient in NQO1 due to enhanced proteasomal degradation of the mutant protein (Siegel et al., 1999, 2001). Epidemiological data from benzene exposed workers has demonstrated an increased risk of developing benzene poisoning (hemotoxicity) with the homozygous NQO1*2 genotype in agreement with the proposed protective role of NQO1 against benzene derived quinones (Rothman et al., 1997). Recently, low NQO1 activity has been associated with increased risk of de novo acute myeloid leukemia in adults (Smith et al., 2001). A hypothesis has been proposed where benzene metabolites phenol and hydroquinone, derived mainly from diet and gastrointestinal flora activity, are causal factors in de novo leukemia (Smith et al., 2001).

Biochemical and immunohistochemical studies revealed that NQO1 is widely expressed in human tissues with high levels of protein detected in epithelium and endothelium (Siegel et al., 1998; Siegel and Ross, 2000). Since NQO1 has been implicated to protect against leukemia we examined human bone marrow for the expression of NQO1. In this study we have performed immunohistochemical staining for NQO1 in paraffin-embedded human bone marrow core biopsies and have examined cultured human bone marrow endothelial cells for NQO1 protein and activity.

2. Materials and methods

2.1. Human tissues and cell lines

Five archival, B-5-fixed, decalcified, paraffin-embedded human bone marrow core biopsies were obtained from the Department of Pathology, School of Medicine, University of Colorado Health Sciences Center. All biopsies were negative for malignancy. All samples were anonymous with no patient identifiers. Immortalized human bone marrow endothelial cell line (HBMEC-60) was obtained from Dr C.E. van der Shoot, University of Amsterdam, Amsterdam, the Netherlands. The HBMEC-60 cell line was grown on fibronectin coated tissue culture ware as previously described (Rood et al., 2000).

2.2. Antibodies

Anti-NQO1 monoclonal antibody (IgG_1) secreting hybridomas (clones A180 and B771) were derived from a BALB-c mouse immunized with purified recombinant human NQO1 protein. Control (non-specific IgG_1 secreting) hybridoma (clone C100) was derived from a BALB-c mouse. Hybridoma tissue culture supernatants were prepared as previously described (Siegel et al., 1999). Anti-NQO1 and control antibodies were diluted 1:1 with 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.4% (v/v) Tween-20 and 5% (w/v) non-fat dry milk (blocking buffer). Detection of NQO1 by IHC was performed using avidin:biotinylated enzyme complex (ABC) methodologies with DAB staining (Vectastain Elite ABC Kit, Vector DAB Substrate Kit, Vector Laboratories, Burlingame, CA).

2.3. Immunohistochemistry

Immunohistochemistry was performed on tissue sections (4 μm) cut from archival paraffin blocks. Briefly, sections were deparaffinized in xylene and rehydrated through graded alcohols to distilled water and then microwaved for two 5 min cycles in 10 mM sodium citrate, pH 6.0. Endogenous peroxidase activity was eliminated by placing sections in 0.1% phenylhydrazine for 20 min. Immunodetection of NQO1 was performed using tissue culture supernatants from mouse hybridoma clones A180 and B771. Negative controls were performed utilizing supernatant from control hybridoma clone C100. Serial sections of each tissue sample were incubated with either anti-NQO1 or control antibodies diluted 1:1 in blocking buffer for 1 h at 27 °C. Immunodetection was performed using a horseradish peroxidase-based Vectastain Elite ABC Kit. 3,3-diaminobenzidine (DAB) and hydrogen peroxide were used as the horseradish peroxidase substrates (brown staining). Sections were then counterstained with hematoxylin. Sections were photographed on a Nikon FX microscope with Kodak Ektachrome 64 film.

2.4. Immunoblot analysis

Immunoblot analysis of NQO1 was performed as previously described (Siegel et al., 1999). HBMEC-60 cells were grown to 80% confluencey in T-75 fibronectin coated flasks. Endothelial cells were released from the culture flask following treatment with trypsin, then washed in PBS and pelleted by centrifugation. Cell pellets were resuspended in 200 μl of 25 mM Tris–HCl, pH 7.4 containing 250 mM sucrose and 1 μM flavin adenine dinucleotide and sonicated on ice for 15 s. Sonicates were centrifuged at $10\,000 \times g$ for 5 min to remove cellular debris. Following centrifugation, the supernatant was recovered and protein concentrations were determined using the method of Lowry (Lowry et al., 1951). Cellular proteins were separated by 12% SDS-PAGE (minigel) and transferred to 0.4 μm PVDF membrane in 25 mM Tris, 192 mM glycine containing 20% (v/v) methanol at 100 V for 1 h. Immunoblot analysis of NQO1 was performed using mouse anti-NQO1 monoclonal antibodies (clones A180/B771). Membranes were incubated in 20 ml of blocking buffer with anti-NQO1 hybridoma tissue culture supernatant diluted 1:200 for 1 h at 27 °C followed by the addition of horseradish peroxidase conjugated goat anti-mouse IgG (1:5000) for 30 min. Protein visualization was performed using enhanced chemiluminescence (ECL) as described by the manufacturer.

2.5. NQO1 activity assay

NQO1 specific activity was determined spectrophotometrically in HBMEC-60 sonicates using dicumarol-inhibitable reduction of 2,6-dichlorophenol-indophenol (DCPIP) as described previously (Benson et al., 1980).

3. Results

Immunohistochemical staining of NQO1 in human bone marrow core biopsies has detected NQO1 protein expression in bone marrow endothelial cells and adipocytes in bone marrow core biopsies samples from five individuals. Immunostaining of NQO1 was detected in endothelial cells of larger arteries as well as in smaller arterioles and sinusoids (Fig. 1). No immunostaining for NQO1 was observed in human bone marrow core biopsies in control experiments when anti-NQO1 antibodies were replaced with non-specific antibodies (Fig. 1A). In addition to endothelial cells immunostaining of NQO1 was also detected in adipocytes (Fig. 1F), however, no staining for NQO1 was detected in hematopoietic cells.

To confirm the presence of NQO1 within bone marrow endothelial cells we examined an immortalized human bone marrow endothelial cell line (HBMEC-60) for NQO1 protein and activity. Immunoblot analysis of HBMEC-60 cells confirmed the presence of NQO1 protein (Fig. 2). NQO1 specific activity was measured in HBMEC-60 cells at 617 ± 8 ($n = 3$) nmoles DCPIP per min per mg protein. These data confirm that HBMEC-60 cells express NQO1 at levels comparable to what has been measured previously in epithelial cell lines.

4. Discussion

Immunohistochemical staining of NQO1 in human bone marrow core biopsies has revealed that NQO1 is expressed in bone marrow endothelial cells. This is in agreement with immunohistochemical studies in other human tissues that have shown NQO1 expression in endothelium. NQO1 has been detected in vascular endothelium of many human tissues that we have examined (Siegel et al., 2001). NQO1 has also been detected in specialized endothelial cells including corneal endothelium, (Schelonka et al., 2000) and alveolar capillary endothelium (Siegel et al., 1998). The absence of immunostaining of NQO1 in hematopoietic cells is in agreement with earlier experiments that failed to detect NQO1 protein in Ficoll-purified human bone marrow mononuclear cells or purified $CD34^+$ hematopoietic progenitor stem cells (Moran et al., 1999).

NQO1 expression has been shown to be protective against benzene-derived quinones and epidemiological evidence suggests that decreased or absent NQO1 activity results in an increased risk

of benzene or chemotherapy-induced leukemias as well as de-novo leukemias in children and adults (Rothman et al., 1997; Larson et al., 1999; Wiemels et al., 1999; Smith et al., 2001). The mechanisms whereby NQO1 might protect against leukemogensis have been puzzling since NQO1 could not be detected in human bone marrow cell aspirates and purified human bone marrow progenitor cell populations (Moran et al., 1999) even from individuals carrying the NQO1*1/*1 (wild type) genotype. In the case of benzene exposure, we offered a potential explanation that benzene metabolites could induce NQO1 in human bone marrow cells but increased NQO1 activity was not observed in cells from individuals homozygous for the NQO1*2 polymorphism (Moran et al., 1999), presumably due to rapid proteasomal degradation of the mutant NQO1 protein (Siegel et al., 2001). In this manuscript, we demonstrate that NQO1 is present in human bone marrow but is contained in cells that are not readily removed by aspiration techniques such as bone marrow endothelial cells. The increased risk of de-novo and chemical induced leukemias associated with the NQO1*2 polymorphism may, therefore, reflect a lack of NQO1 activity in bone marrow endothelial cells

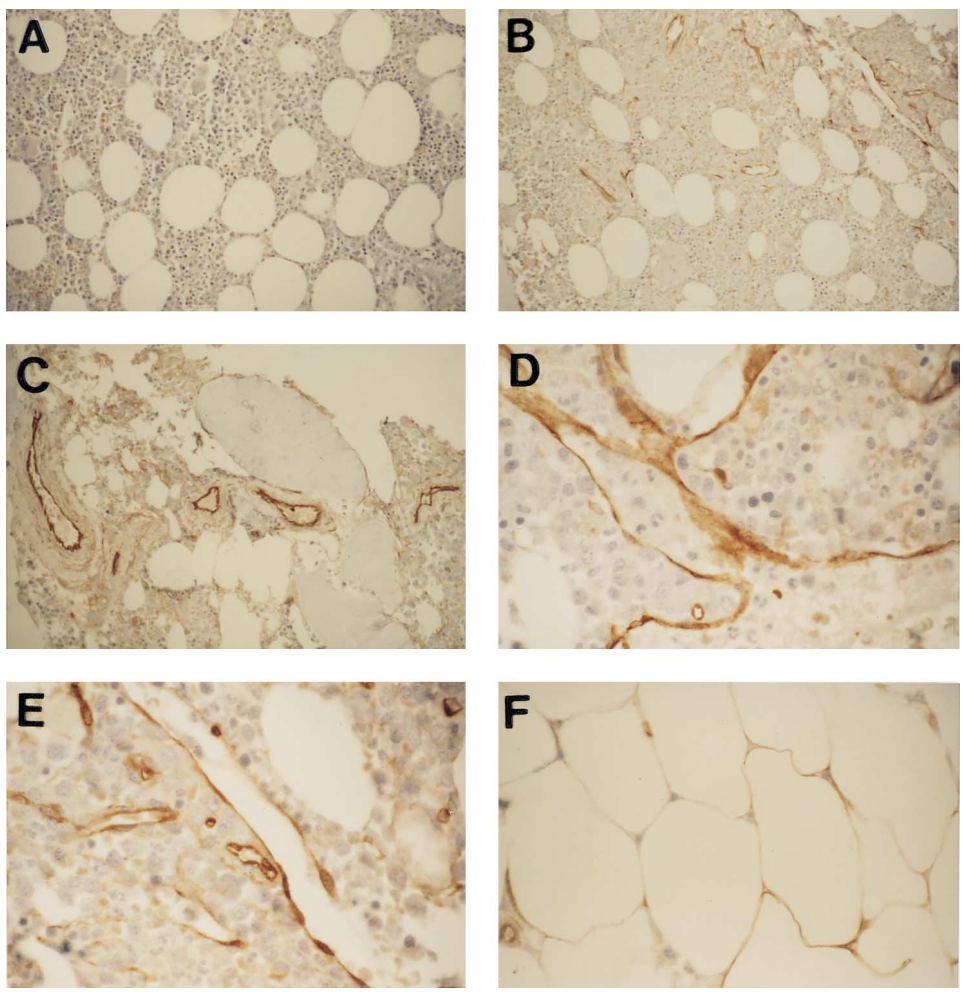

Fig. 1. Immunoperoxidase staining (DAB, brown) of NQO1 in human bone marrow core biopsies. (A) Control antibody; (B) Anti-NQO1 antibody; (C) NQO1 immunostaining of endothelium in larger blood vessels; (D, E) NQO1 immunostaining of endothelium in bone marrow sinusoids; (F) NQO1 immunostaining in bone marrow adipocytes.

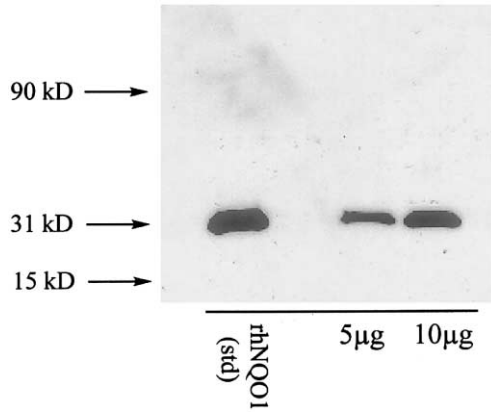

Fig. 2. Immunoblot analysis of NQO1 protein expression in cultured human bone marrow endothelial cells. Cytosol from immortalized human bone marrow endothelial cell line HBMEC-60 was examined for NQO1 protein expression by immunoblot analysis as described in the Materials and Methods. rhNQO1; recombinant human NQO1 standard (5 ng).

and increased susceptibility to chemical and environmental insults.

The expression of NQO1 in bone marrow endothelium is consistent with this enzyme playing a role in protection against leukemagenesis. Bone marrow endothelial cells participate in the maturation of hematopoietic progenitor stem cells through a combination of adhesion molecule interactions and cytokine secretions (Voermans et al., 2000; Mohle et al., 1998). The expression of NQO1 in bone marrow endothelial cells may offer protection against phenolic-derived quinones generated within the endothelial cell or via the surrounding myeloperoxidase-rich hematopoietic progenitor stem cells (Schattenberg et al., 1994). Alternatively, other NQO1-mediated protective mechanisms may also be playing a role. NQO1 has recently been reported to stabilize p53 (Asher et al., 2001), and such a stabilizing effect would presumably be absent in individuals carrying the NQO1 *2/*2 genotype due to the absence of NQO1 protein.

In summary, immunohistochemical analysis of human bone marrow core biopsy specimens showed the presence of NQO1 in endothelial cells and adipocytes. Substantial levels of NQO1 were also detected in an immortalized human bone marrow endothelial cell line. These data suggest that the increased risk of chemical and de novo leukemia that has been associated with the NQO1*2 polymorphism may reflect a lack of NQO1 in bone marrow endothelial cells resulting in increased susceptibility of these cells to environmental damage.

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