Advances in Brief

Immunohistochemical Detection of NAD(P)H:Quinone Oxidoreductase in Human Lung and Lung Tumors

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Abstract

NAD(P)H:quinone oxidoreductase (NQO1) is a flavoenzyme that catalyzes the two-electron reduction of quinones and related compounds. With the use of biochemical assays, NQO1 has been shown to be overexpressed in many types of cancer, including non-small cell lung cancer (NSCLC). NQO1 can bioactivate antitumor quinones such as mitomycin C, and new quinone-based drugs are currently being developed to target this enzyme in tumors such as NSCLC. Because there is no information on the cell-specific expression of NQO1 in lung, the purpose of this study was to examine the expression of NQO1 in human NSCLC, small cell lung cancer, carcinoid lung tumors, and normal lung using immunohistochemistry. A high level of NQO1 protein expression was detected by immunohistochemistry in NSCLC (adenocarcinoma, squamous cell carcinoma, and bronchoalveolar carcinoma), but no NQO1 protein could be detected in small cell lung cancer or carcinoid lung tumors. In addition, NQO1 protein expression was examined by immunohistochemistry in normal lung tissue. A high level of NQO1 protein expression was detected by immunohistochemistry in normal lung respiratory epithelium, with the highest levels of expression observed in ciliated columnar epithelial cells. Significant amounts of NQO1 protein were also detected in the vascular endothelium and adipocytes. These data demonstrate that NQO1 is overexpressed in NSCLC. Cells in normal lung also contain marked NQO1 protein and may be damaged by drugs activated by NQO1. These data validate NSCLC as a target for NQO1-directed agents and suggest that the potential for lung toxicity be considered in the preclinical development of quinone-based antitumor drugs.

Introduction

NQO1 (DT-diaphorase, EC 1.6.99.2) is a flavoenzyme catalyzing NADH- or NADPH-dependent two-electron reduction of a broad range of substrates. Whether reduction by NQO1 results in activation or deactivation of quinones depends upon the properties of the hydroquinone generated. Evidence suggests that simple quinones, such as benzoquinone and naphthoquinone, are deactivated following reduction by NQO1 (1, 2). In contrast to the role of NQO1 in detoxifying quinones, NQO1 has also been shown to play a role in the activation of many quinone antitumor compounds via reduction to their hydroquinone forms. The hydroquinone may autoxidize to produce reactive oxygen species or undergo rearrangement to produce a reactive alkylating species. NQO1 can bioactivate many antitumor quinones such as mitomycin C (3, 4), EO9 (5), streptonigrin (6), MeDZQ (7), and diaziqune (8) via the production of reduction-oxidation-labile hydroquinones and/or reactive alkylating species. The ability of NQO1 to activate these agents has implicated NQO1 as a target enzyme for quinone antitumor drug design (9).

Materials and Methods

Lung Samples. Normal lung and lung tumors were obtained from the University of Colorado Health Sciences Center lung tissue bank. All samples were genotyped as either wild type or heterozygous for the NQO1 699C-to-T mutation using PCR-RFLP with restriction endonuclease Hinfl and genomic DNA

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3 The abbreviations used are: NQO1, NAD(P)H:quinone oxidoreductase
1; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer;
TBSTM, 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.2% Tween 20, and
5% nonfat dry milk; DAB, 3,3-diaminobenzidine.
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Genotyping for the C-to-T mutation was obtained from either blood or lung tissue, as described previously (18). Genotyping for the C-to-T mutation was performed on tissue samples because previous data have shown that cell lines genotyped as homozygous for the C-to-T mutation have decreased NQO1 protein expression (18).

### Anti-NQO1 and Control Monoclonal Antibodies

Anti-NQO1 monoclonal antibody (IgG1)-secreting hybridomas (clones A180 and B771) were derived from a BALB-c mouse immunized with purified recombinant human NQO1 protein. Antibodies from these hybridoma clones will react with both wild-type and mutant NQO1 proteins (18). These antibodies, however, do not cross-react with purified human NQO2. A control (nonspecific IgG1 secreting) hybridoma (clone C100) was derived from a BALB-c mouse. All hybridoma cell lines were grown in spinner flasks in RPMI containing 50 units/ml penicillin, 50 μg/ml streptomycin, 1% l-glutamine (Life Technologies, Inc., Gaithersburg, MD), and 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) in 5% CO2 at 37°C to a concentration of 10⁶ cells/ml. Hybridoma tissue culture supernatants were prepared by centrifugation at 1800 rpm for 10 min and then stored at −80°C. Prior to use, supernatants were centrifuged at 14,000 rpm for 5 min.

### Immunohistochemistry

Lung sections (4 μm) were cut from archival paraffin blocks. Sections were heated to 80°C for 30 min and then deparaffinized in xylene and rehydrated through graded alcohols to distilled water. Sections were then placed in a 10 mM citric acid solution (pH 6.0) and microwaved for two 3-min cycles. Sections were cooled to room temperature and then transferred to PBS for 10 min. Endogenous peroxidase activity was eliminated by placing sections in 3% hydrogen peroxide for 20 min. Sections were rinsed in PBS for 10 min and then blocked in 5% horse serum in TBSTM for 30 min. Immunodetection of NQO1 was performed using tissue culture supernatants from hybridoma clones A180 and B771 mixed 1:1 and then further diluted with an equal part of TBSTM. Negative controls were performed using tissue culture supernatant from control hybridoma clone C100 diluted with an equal part of TBSTM. Serial sections of each tissue sample were incubated with either anti-NQO1 or control antibodies. Sections were incubated with 1 ml of diluted hybridoma supernatant for 30 min at 27°C. Sections were washed in TBSTM for three 10-min cycles. Immunodetection was performed using a horseradish peroxidase-based Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). Biotinylated horse antimouse IgG (H+L) secondary was diluted 1:200 in TBSTM containing 3% horse serum, and 1 ml of diluted secondary antibody was added to each section for 30 min. Sections were washed in TBSTM as described above. Avidin-horseradish peroxidase complex was prepared in TBSTM as described by the manufacturer, and 1 ml of complex was added to each section for 30 min. Sections were washed in TBSTM as described above, followed by a 1-min wash in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.2% Tween 20. DAB and hydrogen peroxide (DAB Staining Kit; Vector Laboratories) were used as the horseradish peroxidase substrates. DAB/hydrogen peroxide solution was prepared according to the manufacturer, and 1 ml of solution was added to each section for 5–7 min. Sections were then rinsed in distilled water, counterstained with hematoxylin or toluidine blue, dehydrated, and mounted. Sections were photographed using a Nikon FX microscope with Agfa RSX-50 film. The intensity of NQO1

### Table 1 Immunostaining of NQO1 in human lung tumors

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Tumor type</th>
<th>Immunostaining of tumor for NQO1</th>
<th>Intensity of immunostaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>2216bcd</td>
<td>Adenocarcinoma</td>
<td>Positive (95)</td>
<td>+4 (cytoplasmic)</td>
</tr>
<tr>
<td>2326cde</td>
<td>Adenocarcinoma</td>
<td>Positive (90)</td>
<td>+3 (cytoplasmic)</td>
</tr>
<tr>
<td>2357f</td>
<td>Adenocarcinoma</td>
<td>Positive (95)</td>
<td>+4 (cytoplasmic)</td>
</tr>
<tr>
<td>5258h</td>
<td>Bronchioloalveolar</td>
<td>Positive (75)</td>
<td>+3-4 (cytoplasmic)</td>
</tr>
<tr>
<td>5229de</td>
<td>Squamous</td>
<td>Positive (80)</td>
<td>+4 (cytoplasmic)</td>
</tr>
<tr>
<td>508f</td>
<td>Squamous</td>
<td>Positive (95)</td>
<td>+4 (cytoplasmic)</td>
</tr>
<tr>
<td>520e</td>
<td>Squamous</td>
<td>Positive (60)</td>
<td>+3 (cytoplasmic)</td>
</tr>
<tr>
<td>5243cde</td>
<td>Squamous</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>5072cde</td>
<td>Small cell</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>5116cde</td>
<td>Small cell</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>5092cde</td>
<td>Small cell</td>
<td>Negative</td>
<td>Tumor negative, +4 staining of normal epithelium</td>
</tr>
<tr>
<td>3304h</td>
<td>Carcinoid</td>
<td>Negative</td>
<td>Tumor negative, +4 staining of normal epithelium</td>
</tr>
<tr>
<td>5433h</td>
<td>Carcinoid</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>2665h</td>
<td>Carcinoid</td>
<td>Negative</td>
<td></td>
</tr>
</tbody>
</table>

*Numbers in parentheses represent percentage of tumor cells staining positive for NQO1.
Female.
Male.
Chemotherapy prior to specimen collection.
No medical history available.
Nonsmoker.
No smoking history available.

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4 S. Chen and D. Siegel, unpublished results.
imunostaining was scored as 0 (negative), 1 (very weak), 2 (weak), 3 (strong), or 4 (very intense). Intensity was judged relative to normal respiratory epithelium (+4) within the sample.

Results

Immunohistochemical Analysis of NQO1 in Human Lung Cancers. Immunohistochemical staining using monoclonal antibodies directed against human NQO1 was performed on formalin-fixed, paraffin-embedded sections of human NSCLC (adenocarcinoma, squamous cell carcinoma, and bronchoalveolar carcinoma), SCLC, and carcinoid lung tumors. NQO1 was detected in eight of nine NSCLC samples (Table 1); immunostaining was not observed in one squamous cell carcinoma. Immunostaining was not observed in control sections when nonspecific mouse monoclonal antibodies were substituted for the primary antibody (data not shown). Immunostaining revealed that NQO1 was present in tumors cells but not the surrounding lymphoid cells or supporting stroma (Fig. 1, A–C). NQO1 was detected in vascular endothelium of some NSCLC (Fig. 1, A, C, and D). Immunostaining for NQO1 was primarily cytoplasmic in both tumor cells and endothelium. The intensity of immunostaining for NQO1 (+3–+4) suggests that NSCLCs contain high levels of NQO1 and that NQO1 expression was relatively homogeneous throughout the tumors. In contrast to NSCLC, no immunostaining for NQO1 was observed in tumor cells from SCLC (0 of 3) or carcinoid lung tumors (0 of 3; Table 1 and Fig. 1, D and E). Positive immunostaining for NQO1, however, was observed in vascular endothelium in some SCLCs and carcinoid lung tumors and attached normal respiratory epithelium (Fig. 1D, arrow). These data demonstrate that NQO1 is expressed in NSCLC but not in SCLC or carcinoid lung tumors.

Immunohistochemical Analysis of NQO1 in Normal Human Lung. Immunohistochemical staining for NQO1 was performed on formalin-fixed, paraffin-embedded sections of

Fig. 1  Immunoperoxidase staining (DAB) of formalin-fixed, paraffin-embedded human lung tumors using monoclonal antibodies to human NQO1. Sections were counterstained with hematoxylin. A, adenocarcinoma; B, squamous cell carcinoma; C, bronchoalveolar carcinoma; D, SCLC, with attached section of respiratory epithelium (arrow); E, carcinoid lung.
Detection of NQO1 in Human Lung and Lung Tumors

Table 2 Immunostaining of NQO1 in normal lung

<table>
<thead>
<tr>
<th>Location</th>
<th>Immunostaining for NQO1</th>
<th>Intensity of immunostaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory bronchi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciliated columnar</td>
<td>Positive (100)</td>
<td>+4 (membrane, cytoplasmic)</td>
</tr>
<tr>
<td>Goblet</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>Positive (10)</td>
<td>+2 (cytoplasmic)</td>
</tr>
<tr>
<td>Stromal</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Lymphoid</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Neural</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Cartilage</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Adipose</td>
<td>Positive (70)</td>
<td>+4 (cytoplasmic)</td>
</tr>
<tr>
<td>Bronchioles</td>
<td>Positive (100)</td>
<td>+4 (membrane, cytoplasmic)</td>
</tr>
<tr>
<td>Goblet</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>Positive (10)</td>
<td>+2 (cytoplasmic)</td>
</tr>
<tr>
<td>Clara</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Alveoli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>Positive (10)</td>
<td>+2 (cytoplasmic)</td>
</tr>
<tr>
<td>Type II</td>
<td>Positive (5)</td>
<td>+2 (cytoplasmic)</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Smooth muscle</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Lymphoid</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Macrophage</td>
<td>Positive (30)</td>
<td>+4 (cytoplasmic)</td>
</tr>
<tr>
<td>Vascular endothelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large vessel</td>
<td>Positive (90)</td>
<td>+3-4 (cytoplasmic)</td>
</tr>
<tr>
<td>Capillaries</td>
<td>Positive (80)</td>
<td>+3-4 (cytoplasmic)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses represent percentage cells immunostaining positive for NQO1 from three separate normal lung samples.

normal human lung from both smokers and nonsmokers. There were no apparent differences in NQO1 immunostaining in normal lung tissue between smokers and nonsmokers. NQO1 protein was detected in respiratory epithelium, vascular endothelium, and adipocytes (Table 2 and Fig. 2, A–H) in all samples. Immunostaining was not observed in control sections when nonspecific mouse monoclonal antibodies were substituted for the primary antibody (not shown). In the respiratory epithelium, columnar epithelial cells immunostained positive (+4), whereas immunostaining was detected in only a few basalar cells (+2; Fig. 2, B and D). No immunostaining was detected in goblet cells (Fig. 2, A and B). Immunostaining of columnar epithelial cells was cytoplasmic and extended on to the ciliated surface (Fig. 2, B and D). In the epithelium of the alveolar ducts and alveoli, immunostaining (+2) was observed in some type I and II pneumocytes (Fig. 2G). NQO1 protein was detected in vascular endothelium with immunostaining (+3–+4) observed in most vessels and capillaries (Fig. 2, E–G). No immunostaining was observed in fibroblasts, smooth muscle, neural, cartilage, or lymphoid tissues in normal lung. Immunostaining was also detected in adipocytes (+4; Fig. 2H) and some macrophages.

Discussion

This study has shown by immunohistochemistry that NSCLC express high levels of NQO1, whereas SCLC and carcinoid lung tumors have very low levels of NQO1 expression. This confirms previous experiments with human lung tumors using biochemical assays for NQO1. Previously, NQO1 has been shown to be overexpressed in many primary tumors, including lung, relative to uninvolved tissue (12). This work was extended by us to measure NQO1 activity in different classes of lung tumors. We demonstrated that there was high NQO1 enzyme activity in NSCLC and cell lines, but, in SCLC and cell lines, NQO1 activity was either very low or nondetectable (17). The high levels of NQO1 protein detected by immunohistochemistry in NSCLC confirms that this protein may be a useful target enzyme to catalyze the bioreductive activation of quinone antitumor agents in these tumors. It has been shown previously that NQO1 can bioactivate mitomycin C (3, 4) and mitomycin C analogues (19), diaziquone (8) and related aziridinyl benzoquinones (7), streptonigrin (6), the indoloquinone EO9 (5), and the dinitrophenylaziridine CB1954 (20).

An unexpected observation was that of high levels of NQO1 expression in the respiratory epithelium. The high levels of NQO1 in the respiratory epithelium suggest that NQO1 may play a role in protecting the epithelium. Pulmonary exposure to quinoid compounds can occur directly from a number of sources, including incomplete combustion, industrial process, and cigarette smoke. A prevalent constituent of cigarette smoke is 1,4-benzoquinone (21, 22), which is considered to play a critical role in hematoxicities following benzene exposure (23). The reduction of 1,4-benzoquinone to hydroquinone by NQO1 is considered a detoxification pathway because hydroquinone can undergo conjugation and excretion (24). The high levels of NQO1 detected by immunohistochemistry in epithelium, endothelium, and adipocytes are consistent with an antioxidant role for NQO1. Endogenous quinones such as a-tocopherol quinone and coenzyme Q derivatives have recently been shown to be substrates for NQO1, and reduction of these compounds by NQO1 results in the formation of hydroquinones with excellent antioxidant properties (25, 26). Reduced forms of a-tocopherol quinone and coenzyme Q are potent inhibitors of lipid peroxidation (25, 26).

NQO1 expression in normal respiratory epithelium and endothelium may present a problem for NQO1-directed agents, and lung toxicities need to be monitored in preclinical studies. The low levels of NQO1 in basal cells of the respiratory epithelium, however, suggests that the high-NQO1-containing epithelium could be regenerated if damage occurred following treatment with NQO1-directed agents. Immunohistochemical studies of NQO1 expression in rat lung have shown that immunostaining of the respiratory epithelium is similar between humans and rats (data not shown). Studies are underway to evaluate the rat as a model to examine the potential toxicity of NQO1-directed agents on respiratory epithelium.

The high levels of NQO1 protein detected by immunohistochemistry on the ciliated surface of the respiratory epithelium suggest that NQO1 may be secreted from these cells. Studies are underway to examine whether NQO1 protein is present in secretory granules of ciliated columnar epithelial cells and bronchial lavage fluid. Previous work has shown that NQO1 could be detected in saliva by activity and immunoblot analysis (27, 28). The role of a secreted NQO1 protein and whether reducing cofactors essential for NQO1 activity will be present in the mucosal fluid remains to be determined.

In summary, immunohistochemical detection of NQO1 in lung tumors has demonstrated high levels of NQO1 expression in NSCLC but not SCLC or carcinoid lung tumors. In addition,
Fig. 2 Immunoperoxidase staining (DAB) of formalin-fixed, paraffin-embedded normal human lung tissue using monoclonal antibodies to human NQO1. Sections were counterstained with toluidine blue (A and B) or hematoxylin (C–H). A–D, respiratory epithelium; E, pulmonary artery; F, pulmonary arteriole; G, alveolar endothelium; H, adipocytes.
this work has shown, for the first time, that NQO1 was present in normal lung but highly localized to epithelial cells and, to a lesser extent, endothelial cells and adipocytes.

References
Immunohistochemical detection of NAD(P)H:quinone oxidoreductase in human lung and lung tumors.

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