Cigarette Smoking Is a Determinant of DT-Diaphorase Gene Expression in Human Non-Small Cell Lung Carcinoma

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ABSTRACT

The levels of NAD(P)H:quinone-acceptor oxidoreductase (EC.1.6.99.2) (DT-diaphorase) mRNA and enzyme activity have been studied in paired human normal lung and non-small cell lung tumor samples from patients with a history of cigarette smoking. There were significantly higher levels of DT-diaphorase mRNA (1.2 kilobases) in lung tumor compared to normal lung tissue of patients who had stopped smoking more than 6 months before surgery, with relative values (normalized to β-actin mRNA) of 29.6 ± 7.8 (SE) in the lung tumor compared to 11.7 ± 2.2 in normal lung tissue (P < 0.05). There was no significant difference in DT-diaphorase mRNA between lung tumor and normal lung tissue of subjects who were smokers at the time of surgery, with values of 16.5 ± 2.1 and 15.3 ± 2.5 (P > 0.05), respectively. DT-diaphorase enzyme activity in normal and tumor lung tissue was positively correlated with DT-diaphorase mRNA (r = 0.908, P < 0.01). The results of the study suggest that DT-diaphorase does not function as an inducible protectant enzyme in human lung against oxidant species and carcinogens present in cigarette smoke. Metabolism of some anticancer drugs by DT-diaphorase can alter their activity. Differences in DT-diaphorase between lung tumors of smokers and past smokers might alter the response to these drugs.

INTRODUCTION

NAD(P)H:quinone-acceptor oxidoreductase (EC.1.6.99.2), also frequently known by its older name DT-diaphorase (1), is a ubiquitous, predominantly cytosolic flavoenzyme with the unique property among NAD(P)H quinone reductases of catalyzing the obligatory two-electron, rather than one-electron, reduction of quinone substrates (1, 2). In humans, DT-diaphorase activity is encoded by four genetic loci (3). The majority of DT-diaphorase activity in human tissue is a 274-amino acid protein and is the product of the dioxin-inducible NQO1 gene which has 84% homology to rat NQO1 DT-diaphorase (4–6). A second non-dioxin-inducible human DT-diaphorase cDNA3 has been cloned, termed NQO2, with 54% homology to NQO1 cDNA, which codes for a 231-amino acid protein (7).

The transcriptional regulation of DT-diaphorase activity is complex. There are at least two cis-acting 5′-flanking promoter elements, one similar to the xenobiotic response element for the cytochrome P450 CYP1A1 gene (8, 9), the other similar to the antioxidant response element of the glutathione S-transferase Ya subunit gene (10) which binds the transcription proteins Jun-D and c-Fos (11). A trans-acting negative regulatory effector gene for DT-diaphorase and other oxidant stress enzymes has recently been identified in mice (12).

DT-diaphorase has been suggested to protect cells against oxidant stress and carcinogenic quinones, and in animals and cultured cells DT-diaphorase activity increases in response to such stimuli (13, 14). Recent interest in DT-diaphorase has also focused on its possible role as a modulator of anticancer drug activity (15). Anticancer drugs that are activated by DT-diaphorase include mitomycin C (16), the dinitrophenylaziridine CB 1954 (18), and the indoloquinone E09 (19). Anticancer drugs reported to be inactivated by DT-diaphorase are trenimon (20) and mitoxantrone (21). Variations in the levels of DT-diaphorase in tumors and normal tissues might lead to differences in the response to these drugs.

We have previously reported that DT-diaphorase activity is elevated in some human primary tumors compared to paired normal tissue (22). The increase in activity could be large, up to 120-fold in one lung adenocarcinoma. We found that subjects who smoked cigarettes had significantly lower DT-diaphorase activity in lung and colon tumors compared to subjects who had stopped smoking cigarettes at least 6 months before surgery, or to non-smokers, but there was no effect on DT-diaphorase activity in the corresponding normal tissue. The effect of cigarette smoking on DT-diaphorase activity in lung was intriguing because the levels of some other putative carcinogen protective enzymes, such as UDP-glucuronosyl transferase, epoxide hydrolase and arylhydrocarbon hydroxylase, have been reported to increase in the lung tissue of smokers (23). In our study, we were not able to determine whether the decrease in DT-diaphorase activity in lung tumors of cigarette smokers was a consequence of the direct inhibition of DT-diaphorase activity by a constituent of cigarette smoke such as benz(a)pyrene metabolites, which are known to inhibit DT-diaphorase activity (24), or to a decrease in DT-diaphorase gene expression.

In the present study, we have examined the effects of cigarette smoking and other patient factors on DT-diaphorase mRNA and enzyme levels in paired normal and human lung tissue. We found that cigarette smoking is a determinant of DT-diaphorase mRNA levels in human lung tumors.

MATERIALS AND METHODS

Human Tissue. Human lung tumor and paired normal lung tissue of subjects undergoing surgery for removal of the tumor were collected from the surgical pathology laboratory within 1 h of removal and immediately frozen and stored in liquid nitrogen. The use of the human tissue had the approval of the Institutional Review Board. Patient characteristics were obtained from medical records. Smoking history was classified as in our previous study of DT-diaphorase activity (22) as follows: non-smokers, no recorded tobacco use (there were no patients in this category); past-smokers, subjects who had smoked ≥ 0.5 packs of cigarettes/day for 20 years to 6 months before surgery; smokers, subjects who smoked ≥ 0.5 packs of cigarettes/day for at least 6 months before the time of surgery.

DT-Diaphorase mRNA. Approximately 1 g of frozen human lung tumor or normal lung tissue was homogenized using a Polytron tissue blender (PT10/35, Kinematica AG, Lucerne, Switzerland) in 6 ml of 4 M guanidinium isothiocyanate, and total RNA was prepared using the isothiocyanate/cesium chloride gradient protocol of Davis et al. (25). Total RNA from SW480 human colon adenocarcinoma cells prepared in the same way was used as a control. Thirty μg total RNA were electrophoresed on a formaldehyde/1% agarose gel and transferred to a Nitran membrane (Schleicher and Schuell, Keene, NH) for blotting. A rat DT-diaphorase cDNA clone pDTD55 (26) was obtained from Dr. C. B. Pickett (Merck Frost, Canada) and used as a DNA template in the polymerase chain reaction to generate a 744-base pair probe. Oligos 2131 (5′-GGCTGCTGTGGAGGCTCTGAAG-3′) and 2132 (5′-CTAGCTTTGCATCGTGTTGC-3′) were used to amplify a fragment of pDTD55 between base pairs 120 and 860. It should be noted that the 5′-end of pDTD55 upstream from base pair 85 has been found to contain a cDNA cloning artifact (6). This region was not amplified in our probe. The RNA blot was hybridized

1 The abbreviation used is: cDNA, complementary DNA.

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2 To whom requests for reprints should be addressed, at Arizona Cancer Center, 1515 N. Campbell Avenue, Tucson, az 85724.
3 The abbreviation used is: cDNA, complementary DNA.
with the [32P]-α-dCTP labeled DT-diaphorase cDNA probe. Prehybridization and hybridization were carried out at 65°C in 1% bovine serum albumin, 0.5 M sodium chloride-0.33 M sodium citrate for 10 min, and at 65°C in 0.25 M sodium phosphate (pH 7.2)-2% sodium dodecyl sulfate-1 mM EDTA for 30 min. The DT-diaphorase probe was removed from the blot, and the blot was re-hybridized with a [32P]-α-dCTP β-actin cDNA probe. Autoradiograms of the blot were scanned using a video densitometer (Model 620; Bio-Rad, Richmond, CA). DT-diaphorase mRNA levels in the cells were corrected for unequal loading and/or transfer using β-actin mRNA.

DT-Diaphorase Activity. Approximately 1 g of tissue was pulverized in liquid nitrogen and then homogenized at 4°C in 8 volumes 50 mM Tris (pH 7.8) using a Polytron tissue blender (PT 10/35; Kinematica AG). The homogenate was sonicated 3 × 10 s (Ultrasonic Processor XL; Heat Systems, Farmingdale, NY) and centrifuged at 10,000 × g for 10 min to remove unhomogenized tissue. The supernatant was assayed spectrophotometrically for DT-diaphorase activity as the dicumarol-inhibitable reduction of dichloroindophenol at 600 nm as described previously (27). Protein was measured by the method of Lowry et al. (28) using crystalline bovine serum albumin as a standard.

Statistical Analysis. Statistical analysis was by the paired and nonpaired t test (29). The data was first log transformed to obtain a normal distribution. A P < 0.05 was considered significant.

RESULTS

Northern blotting showed four bands of DT-diaphorase mRNA in both normal human lung and tumor tissue (Fig. 1). The major DT-diaphorase mRNA band was 1.2 kilobases (band 3) with smaller amounts of a 2.7-kilobase band (band 4) and two bands < 1.2 kilobases (bands 1 and 2) (Fig. 2). Jaiswal (30) has identified 4 potential polyadenylation sequences on the human NQ01 gene and Jaiswal et al. (5) found three species of DT-diaphorase mRNA (1.2, 1.7, and 2.7 kilobases) in human hepatoblastoma cells and reported that similar bands are present in normal human liver. The latter observation is interesting because human liver has very low DT-diaphorase activity compared to other human tissues (22, 31). We only occasionally found a weak 1.8-kilobase mRNA band in some human lung tissues. We found 1.2- and 2.7-kilobase species in SW480 human colon carcinoma cells (Fig. 1) and HT-29 human colon carcinoma but not the smaller mRNA bands found in the human normal lung and primary lung tumor tissue. The levels of the 1.2-kilobase DT-diaphorase mRNA band (band 3) for individual subjects are shown in Table 1. There was a significant positive correlation between the levels of 1.2-kilobase DT-diaphorase mRNA and DT-diaphorase activity for all the tissues (normal lung and lung tumor) examined (r = 0.908; P < 0.01). However, in individual lung samples similar levels of DT-diaphorase mRNA could be associated with different levels of DT-diaphorase activity (e.g., in Table 1 compare subject 7, normal lung and tumor tissue; or subject 29, normal lung; and subject 32, lung tumor). The reasons for this are not entirely clear, but factors apart from mRNA levels may play a greater role in determining DT-diaphorase activity in tissues from these subjects. Subjects who had ceased smoking cigarettes at least 6 months before surgery showed significantly higher levels of 1.2-kilobase DT-diaphorase mRNA in lung tumor compared to paired normal lung tissue (Table 1). Patients who were cigarette smokers at the time of surgery showed no significant increase in 1.2-kilobase DT-diaphorase mRNA in lung tumor compared to the paired normal lung tissue. Cigarette smoking had no significant effect upon DT-diaphorase mRNA in normal lung tissue. The levels of the other DT-diaphorase mRNA bands in normal lung and lung tumor are shown in Table 2. The levels of bands 1 and 2 DT-diaphorase mRNA were not significantly elevated in lung tumor compared to normal lung in either past smokers or smokers. Band 4 (2.7-kilobase) DT-diaphorase mRNA showed a significant elevation compared to normal lung tissue in tumors from past-smokers. The levels of band 4 DT-diaphorase mRNA were also significantly correlated with the levels of band 3 DT-diaphorase mRNA (r = 0.919; P < 0.05). No other patient characteristics besides cigarette smoking, including age, sex, alcohol use, and tumor pathology, were associated with increased DT-diaphorase mRNA or DT-diaphorase activity.

DISCUSSION

The results of the study confirm our previous observation of increased levels of DT-diaphorase activity in lung tumors compared to paired normal lung, of subjects who were not cigarette smokers at the time of surgery, and of no increase in DT-diaphorase activity in the tumors of cigarette smokers (22). We have now shown that the changes in DT-diaphorase activity are associated with changes in
DT-diaphorase mRNA was measured by densitometric scanning of Northern blot autoradiograms and normalized to β-actin mRNA. Values are given for the major 1.2-kb DT-diaphorase mRNA band (band 3) in arbitrary units. Mean values are given ± SE. DT-diaphorase activity was measured in tissue homogenates as the dicumarol-inhibitable reduction of dichloroindophenol. Tissues are classified according to smoking histories of the patients obtained from medical records.

**Table 2** Effect of cigarette smoking on all DT-diaphorase mRNA species in human normal lung and lung tumor tissue

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Age (Yr)</th>
<th>Normal lung</th>
<th>Lung tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DT-mRNA units</td>
<td>DT-activity nmol/min/mg</td>
<td>Pathology</td>
</tr>
<tr>
<td></td>
<td>NA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>A</td>
<td>S</td>
</tr>
<tr>
<td>Past-smokers</td>
<td>12</td>
<td>62</td>
<td>7.2</td>
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<td></td>
<td>15</td>
<td>84</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>76</td>
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</tr>
<tr>
<td></td>
<td>28</td>
<td>72</td>
<td>11.2</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>66</td>
<td>8.0</td>
</tr>
<tr>
<td>Mean</td>
<td>11.7 ± 2.2</td>
<td>13.9 ± 2.2</td>
<td>29.6 ± 7.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Smokers</td>
<td>7</td>
<td>M</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>20</td>
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<td>69</td>
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<tr>
<td></td>
<td>21</td>
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<tr>
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<td>29</td>
<td>M</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>F</td>
<td>66</td>
</tr>
<tr>
<td>Mean</td>
<td>15.3 ± 2.5</td>
<td>16.8 ± 12.1</td>
<td>16.5 ± 2.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Pathology: S, squamous cell; A, adenocarcinoma; L, large cell.

<sup>b</sup> NA, insufficient tissue available for assay.

<sup>c</sup> P < 0.05 by paired t test of normalized log transformed data compared to value for normal lung.

Decreased levels of DT-diaphorase activity in lung tumors of smokers may make these tumors less susceptible than lung tumors of non-smokers to the cytotoxic effects of quinone anticancer drugs that are activated by DT-diaphorase. There have been few studies of the effects of smoking cessation on response to treatment in cancer patients (39). There are reports that smokers with lung cancer and other cancers have a shorter survival compared to past smokers or subjects who stop smoking at the time of surgery (40–44). However, not all of these patients received chemotherapy, and it is not possible to identify a common factor in their treatment that may be affected by smoking. The effects of smoking-induced changes in DT-diaphorase activity on responses to chemotherapy is a topic that requires further investigation.

In summary, we have shown that a decrease in DT-diaphorase mRNA is associated with a decrease in DT-diaphorase activity in non-small cell lung cancer of patients who are cigarette smokers at the time of surgery, compared to patients who had not smoked for at least 6 months before surgery. There was no significant effect of smoking upon DT-diaphorase expression in normal lung tissue.

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