Resistance of Murine Lung Tumors to Xenobiotic-induced Cytotoxicity

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ABSTRACT

Studies were performed to test the hypothesis that urethane-induced murine lung tumors exhibit xenobiologic resistance and alterations in pulmonary cytochrome P-450 enzymes. 1,1-Dichloroethylene, naphtha-

lene, and paraquat were administered to tumor-bearing and control mice to elicit acute lung cytotoxicity, and responses were evaluated in tumors (papillary and solid), uninvolved surrounding tissue, and untreated control lung. 1,1-Dichloroethylene (125 mg/kg, i.p.) and naphthalene (225 mg/kg, i.p.) caused preferential necrosis of Clara cells in control lungs and uninvolved tissue of tumor-bearing lungs. In contrast, papillary and solid tumors were both resistant to 1,1-dichloroethylene-induced cytotoxicity. Paraquat (10, 20 mg/kg, i.v.) elicited Clara cell damage in control lungs and uninvolved lung tissue of tumor-bearing mice, with minor disruption of the alveolar epithelium. Neither papillary nor solid tumors sustained any apparent cell damage from paraquat. Immuno-

louls of P-450 enzymes confirmed constitutive expression of CYP2BI in control lung and uninvolved lung tissue of tumor-bearing mice, but this P-450 enzyme was not detected in either adenomas or carcinomas. Lung CYP1A1 was inducible by β-naphthoflavone in non-tumor-bearing mice and uninvolved tissue of tumor-bearing mice; however, inducibility was decreased in adenomas and abolished in carcinomas. These results demonstrate resistance of lung tumor cells to chemically induced cytotoxicity and diminished expression of cytochrome P-450 enzymes in tumors.

INTRODUCTION

The concept of a new cell phenotype that appears after exposure to chemical carcinogens arose from the studies of Had-

dow (1), who showed that, although carcinogenic polycyclic aromatic hydrocarbons exerted cytotoxic and growth-inhibitory effects on normal tissues, the tumors that they induced were resistant to these alterations. These studies have also led to the hypothesis that "the origin of cancer" must involve the mani-

festation of novel properties within tumor cells that permit growth under conditions that are toxic to normal cells (1). In support of this hypothesis, extensive studies in rodent models of hepatocarcinogenesis have demonstrated the expression of a phenomenon initiated by carcinogens and described as the "resistance phenotype" (2-4). It has further been proposed that this resistance phenotype, which is manifest in a small number of hepatocytes, subsequently undergo clonal expansion and aggressively proliferate to form nodules, whereas growth of the vast majority of nonresistant hepatocytes is inhibited (2, 4). Hyperplastic nodules induced in rat liver by 2-acetylaminoflu-

orene or ethionine were resistant to the acute hepatotoxic effects of carbon tetrachloride and dimethylnitrosamine in vivo, under conditions in which necrosis was evoked in tissues sur-

rounding the nodules as well as in control liver (3). Hepatocytes isolated from livers of rats previously administered aflatoxin B1 and maintained in culture survived subsequent exposure to afla-

toxin B1, whereas cell death occurred in hepatocytes from untreated control rats (5). Confirmation has also been obtained in vitro for the cross-resistance observed in vivo; cell cultures der-

ived from hepatic nodules induced by 2-acetylaminofluorene were resistant to concentrations of aflatoxin B1 that killed cul-

tured normal hepatocytes (3, 6). This phenotypic alteration appears to be constitutive in as much as cell lines derived from resistant hepatocytes demonstrate similar resistance (5).

Carcinogen-induced xenobiologic resistance in liver tumors has in part been ascribed to a distinctive biochemical and metabolic profile that is different from that seen in normal or uninvolved surrounding tissues (7-9). Liver tumor cells have low levels of cytochrome P-450 and other Phase I enzymes involved in biotransformation of xenobiotics and carcinogens (7, 9-15). Cytochrome P-450 content and microsomal enzyme activities such as aminopyrene N-demethylase, aryl hydrocarbon hydrox-

ylase, ethoxyresorufin O-deethylase, and ethoxycoumarin O-de-

ethylase are diminished in hepatic nodules when compared to levels in normal or surrounding liver tissues (7, 9-15), and are even further reduced in large nodules or hepatomas (8, 12-15).

Whereas rat models of hepatocarcinogenesis have been used extensively to investigate xenobiologic resistance, the response of lung tumors to toxicants or carcinogens has received scant attention, despite the availability of well-established animal mod-

els of lung tumorigenesis and the active role of the respiratory system in the metabolism of foreign chemicals. Here we have undertaken to investigate carcinogen-induced xenobiologic resis-

tance in lung tumors, and have used a urethane-induced murine model of lung tumorigenesis. In this model, lung tumors induced by urethane exhibit structures that are either solid or papillary (16, 17). Solid tumors arise in alveolar septae and subsequently proliferate to produce a spherical, compact mass of cells with morphological features characteristic of Type II cells (18). Papillary tumors arise in or adjacent to bronchioles, exhibit a more open tubular pattern, and are lined by columnar epithelial cells characteristic of nonclimated Clara cells (18). Spontaneous lung neoplasms, which are similar in structure to chemically induced tumors, may possess considerable cellular heterogeneity (19). Nonetheless, the solid and papillary classification of adenomas has served as a useful framework upon which to base observations and is used herein.

The potential resistance of lung tumors to cytotoxicity was investigated with the pneumotoxicants, DCE,3 naphthalene, and paraquat. 1,1-Dichloroethylene (20) and naphthalene (21) preferentially damage Clara cells, while paraquat has been re-
ported to target alveolar Type II cells (22, 23). These chemicals were administered to tumor-bearing mice to induce necrosis and cell death in Clara and Type II cells, the predominant cell populations in papillary and solid tumors. The reactivities of these tumors were compared with cellular responses in uninvolved tissues from the same lungs as well as in non-tumor-bearing lungs. In view of the association between decreased P-450 expression and hepatocarcinogenesis, modification in

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3 The abbreviation used is: DCE, 1,1-dichloroethylene.
expression of the major pulmonary cytochrome P-450 enzymes, CYP2B1 and CYP1A1 [defined according to recommended nomenclature (24)], was investigated in benign and neoplastic lung tumors and compared with uninvolved tissues and normal lungs.

These studies demonstrate that lung tumors, irrespective of cell type or structure, are resistant to cytotoxicity induced by DCE, naphthalene, and paraquat. This resistance coincides with a marked reduction in the constitutive expression of CYP2B and the inducibility of CYP1A.

MATERIALS AND METHODS

Treatment of Animals. Strain A/J and Swiss Webster mice of 6 weeks of age were obtained from The Jackson Laboratory (Bar Harbor, ME) and Charles River Canada (St. Constant, Quebec, Canada), respectively. Mice were maintained on a 12-h light/dark cycle and allowed free access to food (Purina Rodent Chow) and water. Mice were administered a single dose of urethane (1 mg/g body weight) by i.p. injection. After tumors were developed 12–16 weeks following urethane administration, groups of 6–8 mice were treated with the following chemicals: DCE (125 mg/kg, i.p.) in corn oil, paraquat (10, 20 mg/kg, i.v.) in saline, and naphthalene (225 mg/kg, i.p.) in corn oil. Mice were killed 24 h after pneumotoxicant treatment.

In experiments to determine CYP1A1 inducibility, mice were given injections of β-naphthoflavone (100 mg/kg; i.p.) in corn oil on each of 2 consecutive days, and killed 48 h after the second dose. The following experimental groups of mice received β-naphthoflavone-treatment: (a) untreated control mice; (b) mice bearing benign tumors (adenomas); and (c) mice bearing malignant tumors (adenocarcinomas). Mice with adenomas were animals that had been treated with urethane 14–16 weeks previously, whereas mice with carcinomas were those that had been treated with urethane 6 months previously. Control groups were treated with corn oil only.

Histopathology. Lung tissues were prepared and processed for histopathology according to procedures detailed in previous studies (25, 26) with the following modification. Tracheal instillation was performed using 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate/HCl buffer, pH 7.4, and tissues were fixed for 24 h at 4°C. Tissues were subsequently processed and embedded in epoxy resin. Tissue sections (1.0 μm) were stained with toluidine blue and examined by light microscopy.

Lungs from naphthalene-treated mice were fixed with Bouin’s fixative by tracheal instillation. Tissues were removed, immersed in fixative for an additional 30 min, and then sectioned into slices; tissue specimens with tumors were fixed for an additional 24 h. Tissue samples were processed and embedded in paraffin, and sections (5 μm) were stained with hematoxylin and eosin for histopathological evaluation.

Preparation of Microsomes. Microsomes were prepared from normal lung tissue, adenomas, carcinomas, and uninvolved tissue of tumor-bearing lungs. Microsomal samples from normal lung tissue were prepared from a single pair of lungs, whereas adenomas were dissected from the lungs of 6 mice and pooled. Lungs with carcinomas were immersed in cold saline and kept on ice, and uninvolved tissue was removed; malignant tumors from 5 mice were pooled for preparation of microsomes.

Tissue samples were homogenized in 3 volumes of a buffer containing 50 mM Tris-HCl, 20% glycerol, 1 mM EDTA, 150 mM KCl, 100 μM dithiothreitol, 1 μM leupeptin, and 1 μl/ml aprotinin, pH 7.4. After a brief sonication (2 × 5 s) and centrifugation at 16,000 × g for 25 min, supernatants were subjected to centrifugation at 100,000 × g for 1 h. Microsomal pellets were resuspended in 200–300 μl of buffer and stored at −70°C. Protein content was determined by the method of Lowry et al. (27).

Cytochrome P-450 Purification, Antibody Production, and Immunoblotting. CYP2B1 (P450b) and CYP1A1 (P450c) were purified from rat liver microsomes as described previously (28, 29). Polyclonal antibodies against these enzymes were raised in rabbits, and were subjected to immunoabsorption chromatography to remove antibodies that cross-reacted with P-450 enzymes in other gene families and subfamilies (30, 31). After immunoabsorption chromatography, anti-1A1 was monospecific, whereas anti-2B1 recognized both 2B1 and 2B2, which are 97% identical in amino acid sequence (28). Immunoblotting of mouse lung microsomes was performed as described previously (32, 33). It must be noted that the lack of positive immunoreactivity for specific P-450 enzymes does not necessarily indicate absence of the proteins, but may be related to the limit of our detection system.

Materials. Urethane, paraquat (methyl viologen, 1,1′-dimethyl-4,4′-bipyridinium dichloride), naphthalene, paraformaldehyde, and β-naphthoflavone were obtained from Sigma Chemical Co. (St. Louis, MO), 1,1-dichloroethylene (>99% purity) was from Aldrich Chemical Co. (Montréal, Québec, Canada), and sodium pentobarbital (Somnotol) was from M.T.C. Pharmaceuticals (Hamilton, Ontario, Canada). Chemicals for electron microscopy were supplied by J.B. EM Services, Inc. (Point Claire-Dorval, Québec, Canada). All other chemicals were of reagent grade and were purchased from standard commercial suppliers.

RESULTS

Histopathological Evaluation of Cytotoxicity. Tumors of both histological types, solid (Fig. 1) and papillary (see Fig. 3A), were present in lungs of mice administered urethane. As expected from previous studies (34), solid tumors predominated in lungs of Strain A/J mice, whereas papillary tumors were more numerous in lungs of Swiss Webster mice. Adenomas at early and late stages of tumor progression were observed for both tumor types, with frequencies being dependent on time elapsed after urethane exposure, as described previously (35). Early in the course of solid tumor formation, foci in the alveolar septae exhibited marked hyperplasia. In these foci, hypercellularity was more pronounced in central than in peripheral areas. Papillary tumors were found either within bronchioles or in an adjacent location. Papillary tumor formation within bronchioles was initially characterized by hypercellularity with marked increases in the number of Clara cells (Fig. 2B) as compared to controls (Fig. 2A). These subsequently lined infoldings or projections of interstitial tissue (Fig. 2C). Papillary tumors that arose adjacent to bronchioles were first observed as discrete

Fig. 1. Solid alveolar tumor induced in murine lung from Strain A/J mouse 14 weeks after administration of urethane. Toluidine blue stain. Original magnification, × 665.
A dose of 125 mg/kg DCE caused Clara cell necrosis in bronchioles of lungs from both murine strains. However, bronchiolar damage was relatively more severe in Swiss Webster than in Strain A/J mice. The Clara cell lesion has been described in detail previously in other strains of mice (20, 36). Structural alterations were not detected in tumors from the same lungs, and this result was obtained irrespective of whether the tumor was papillary (Fig. 3.4) or solid (Fig. 3B), or whether the tumor cells were located centrally or at the periphery of the tumor. Also, no histopathological changes were sustained by solid tumors regardless of the stage of tumor development. However, a difference in response to DCE was observed in hyperplastic bronchiolar epithelium. During the time when hypercellularity was the predominant feature (Fig. 2B), DCE treatment resulted in necrosis of Clara cells (Fig. 3C); in contrast, Clara cells were not damaged when DCE exposure occurred at a time when infoldings of epithelium had formed (Fig. 3D).

Results similar to those found with DCE were obtained when mice were administered a dose of 225 mg/kg naphthalene (Fig. 4). Bronchiolar epithelial lining cells in lungs of non-tumor-bearing mice were severely damaged by naphthalene exposure. In contrast, tumors were not affected and exhibited morphological features similar to those of tumors from mice that were not exposed to naphthalene (Fig. 4A). However, bronchioles in uninvolved portions of tissue surrounding tumors were damaged (Fig. 4B) to a similar extent as found in bronchioles from non-tumor-bearing mice. Epithelial cells were necrotic and many Clara cells were exfoliated from the underlying interstitium (Fig. 4B). Differences in response were not detected between solid and papillary tumors.

Administration of a dose of 10 mg/kg paraquat evoked bronchiolar damage with striking vacuolization of the Clara cells (Fig. 5A). By comparison, alveolar septal damage was mild. Doubling the dose of paraquat to 20 mg/kg exacerbated the bronchiolar injury and caused disruption of the alveolar septum. Neither dose of paraquat elicited cytotoxic reactions in the tumors, regardless of whether they were in early or late stages of tumor formation (Fig. 5, B and C). As was found with DCE, cell responses were not different in central and peripheral portions of the tumor.

In summary, administration of the pneumotoxicants, DCE, naphthalene, or paraquat, resulted in cytotoxic responses in non-tumor-bearing lungs as well as in uninvolved lung tissue of tumor-bearing mice. In striking contrast, cell damage was not observed in papillary or alveolar tumors.

Immunoblotting. Polyclonal antibodies were used to determine the constitutive expression of CYP2B and the inducibility of CYP1A in normal and tumor-bearing mice. The results of these immunoblotting experiments are summarized in Fig. 6. The antibody against rat 1A1 recognized a β-naphthoflavone-inducible protein in mouse lung microsomes (~Mr, 56,000). The antibody against rat 2B1 recognized a Mr ~52,000 protein that was constitutively expressed in mouse lung microsomes (The β-naphthoflavone-inducible mouse lung protein recognized by antibody against rat CYP1A1 is presumably mouse CYP1A1. The identity of the mouse lung protein recognized by antibody against rat CYP2B1 is not known, although CYP2B1 is constitutively expressed in rat lung (32). For convenience, we have assumed that this protein is a CYP2B protein, although data to support such a classification are lacking.). The CYP2B protein was detected in mouse lung microsomes from non-tumor-bearing mice as well as in microsomes from uninvolved tissues of

Fig. 2. Bronchiolar epithelium in murine lung from control and urethane-treated Strain A/J mice. Clara cells (arrowheads) are interspersed among ciliated cells of the bronchiolar epithelium from normal lung (A). Treatment with urethane results in hypercellularity of the epithelial lining, with marked increase in the number of Clara cells (arrowheads; B). Hyperplasia of the epithelium is characterized by folds (arrows) in the epithelial lining (C). Toluidine blue stain. Original magnification, ×265 (A); ×275 (B); ×660 (C).

hyperplastic foci that enlarged during progression. When established, both solid and papillary tumors exhibited distinctive features such as the compact pattern of the former (Fig. 1) versus the open tubular pattern of the latter (Fig. 3A).
Fig. 3. Lungs of Swiss Webster mice administered urethane and DCE (125 mg/kg). Treatment of tumor-bearing mice with DCE does not cause any injury to cells within papillary (A) or solid (B) tumors. Clara cells of the hypercellular epithelial lining are damaged by DCE treatment (arrows; C). DCE treatment has no deleterious effect when folding of the epithelial lining has developed (D). Toluidine blue stain. Original magnification: × 415 (A); × 265 (B); × 415 (C); × 660 (D).

Fig. 4. Lungs of Strain A/J mice administered urethane and naphthalene (225 mg/kg). Naphthalene treatment of tumor-bearing mice causes no structural change in solid tumors (A), but elicits necrosis and exfoliation (arrow) of the bronchiolar epithelium in uninvolved tissue of the same lung (B). Hematoxylin and eosin stain. Original magnification, × 2000 (A and B).

adenoma-bearing lungs. This CYP2B protein was not detectable in microsomes prepared from adenomas. Lung microsomes from non-tumor-bearing mice treated with β-naphthoflavone contained both CYP1A and CYP2B proteins. Both proteins were also detected in microsomes from uninvolved lung tissues of adenoma-bearing mice treated with β-naphthoflavone. However, in the adenoma itself, CYP2B was not expressed, and the inducibility of CYP1A by β-naphthoflavone was diminished compared with uninvolved tissue from the same mice. In carcinomas, CYP2B was not constitutively expressed, and CYP1A was not inducible by β-naphthoflavone. Thus, the formation of tumors results in loss of CYP2B and a progressive diminution of CYP1A inducibility in adenomas and carcinomas.

DISCUSSION

Unique properties characterize the respiratory system that are markedly different from those of the hepatic system. In particular, its cellular diversity is reflected in lung tumors with
differing morphological features belonging to either Clara or Type II cells (18). As well, chemically induced pneumotoxicities affect particular cell populations preferentially. Clara cells are the primary target of several chemical pneumotoxicants, including DCE (20) and naphthalene (21), which were used in the current studies. Less frequently affected, but also susceptible to cytotoxicity, are alveolar Type II cells, which are most severely damaged when rats are exposed to the herbicide, paraquat (22, 23). It is intriguing that the cell types that are the targets of chemically induced cytotoxicity are also the cells implicated in the formation of lung tumors. It remains to be determined whether there are any relationships between the cell constituents of tumors and the susceptibility of the same cell families to cytotoxic injury.

Our histopathological evaluation confirms those of previous studies that have found Clara cells to be preferential targets of cytotoxicity induced by DCE (20) and naphthalene (21). However, our observations in mice show that the pulmonary lesion induced by paraquat differs from that described in rats, a species in which paraquat-induced toxicity has been most studied (22, 37, 38). Whereas alveolar Type II cells are damaged in rat lungs exposed to paraquat, Clara cells are a prominent target in mouse lung (Fig. 5A), with only minor alveolar septal disruption. A lesion primarily involving the Clara cells was also described in mice treated with paraquat i.p. (39). On the other hand, an ultrastructural study found pneumonia to be the primary pulmonary response in mice administered paraquat in drinking water (23). These findings suggest species differences in paraquat toxicity, and further that the route of exposure to paraquat may influence the site of the lesion and/or the ultimate pathological consequences.

In these experiments, mouse lung tumors were found to be resistant to the cytotoxic effects of pneumotoxicants. Although...
normal lung and uninvolved lung tissue of tumor-bearing mice were sensitive to cytotoxicity induced by DCE, naphthalene, and paraquat, lung tumors were resistant to the toxic effects of these chemicals regardless of whether the foci were papillary or solid. However, the “resistant phenotype” differed in one particular respect in hyperplastic foci of alveolar and bronchiolar epithelium: resistance was observed in conjunction with hypercellularity of alveolar epithelium, whereas in the bronchiolar epithelium hypercellularity in itself was not associated with resistance (Fig. 2, A and B) because it was only when the epithelial lining was folded that resistance developed (Fig. 3D). It is thus significant that, in the evolution of solid tumors, no specific structural alteration can be observed to identify a time when resistance appears. However, in papillary tumors, this event is morphologically distinct.

Our studies have found lung tumor cells to be resistant to cytotoxic xenobiotics that are structurally dissimilar and whose mechanisms of action are markedly different. Clara cell injury caused by DCE (40, 41) and naphthalene (42, 43) involves metabolic activation from proximate toxicants to ultimate toxic species within the lung. A different mechanism has been postulated for paraquat; Type II cell damage is attributed to redox cycling reactions involving the “activation” of molecular oxygen to superoxide radicals and other reactive oxygen species (44). Alternatively or in addition, electron transfer from pyridine nucleotides may deplete cellular-reducing components such as reduced nicotinamide-adenine dinucleotide phosphate (45, 46). These findings suggest that lung tumor cells may be different from normal cells in that their capability to generate reactive metabolites is diminished or abolished. However, the deficiency of cytochrome P-450s in tumor foci represents only one of a number of factors that may contribute to the resistance to cytotoxicity.

The basis for xenobiotic resistance in lung tumors has not been elucidated. However, a possible explanation for xenobiotic resistance in hyperplastic liver nodules has been advanced. Available data indicate a relative deficiency within nodules of enzymatic components, including cytochrome P-450, that are known to play a central role in bioactivation of hepatotoxins and hepatocarcinogens (2–4, 7–10). In the case of dimethylnitrosamine and 2-acetylaminofluorene, resistance of pre-malignant hepatocytes within hyperplastic nodules coincides with decreased generation of reactive metabolites capable of binding covalently to DNA, RNA, and protein. This implies that the basis for resistance is related to reduced metabolic activation (3).

Immunohistochemical studies (47) show that CYP2B is constitutively expressed in murine lung cells and does not appear to be inducible by phenobarbital. In contrast, CYP1A1 is not constitutively expressed in mouse lung but is highly inducible by β-naphthoflavone and 3-methylcholanthrene (47). Our experiments in control and β-naphthoflavone-treated mice confirm this pattern (Fig. 6). A similar P-450 profile was found in uninvolved lung tissue in tumor-bearing mice, but a distinct change was evident in adenomas and carcinomas, where CYP2B expression is suppressed so that this otherwise constitutive protein is no longer detectable. Inducibility of CYP1A1, as assessed by its expression after treatment of mice with β-naphthoflavone, decreased with progression of tumorigenesis: CYP1A1 inducibility was reduced in adenomas and abolished in carcinomas. These findings suggest that tumorigenesis is associated with diminished expression of P-450 enzymes in mouse lung. A comparison of these findings in lung tumors with those in liver tumors indicates a similar pattern of lowered P-450 expression (48), with progressive diminution in the expression of individual P-450 enzymes during the course of hepatocarcinogenesis (49, 50). The alterations in P-450 enzymes in liver tumors and in lung tumors in our experiments indicate a general pattern of reduced expression in both model systems, suggesting that similar fundamental processes may be involved in neoplastic transformation.

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