3-Methylindole-Induced Toxicity to Human Bronchial Epithelial Cell Lines

William K. Nichols, Rashmi Mehta, Konstantine Skordos, Katherine Macé, Andrea M. A. Pfeifer, Brian A. Carr, Tamara Minko, Scott W. Burchiel, and Garold S. Yost

Transfected BEAS-2B cells that express different cytochrome P450 enzymes were used to assess whether human bronchial epithelial cell lines are target cells for 3-methylindole (3MI)-induced damage. Four different transfected BEAS-2B lines overexpressing P450s 2A6, 3A4, 2F1, and 2E1 (B-CMV2A6, B-CMV3A4, B-CMV2F1, and B-CMV2E1), respectively, were compared. The B-CMV2F1 and B-CMV3A4 cells were the most susceptible to 3MI-mediated cytotoxicity, measured by leakage of lactate dehydrogenase into the medium after a 48-h incubation. The toxicity was ameliorated by pretreatment with 1-aminobenzotriazole (ABT). Depletion of glutathione with diethylmaleate decreased the cytotoxicity of 3MI to human lung epithelial cell lines. Additional studies clearly demonstrated that a low concentration of 3MI (10 μM) induced apoptosis in BEAS-2B cells that was measured by DNA fragmentation, and apoptosis was inhibited by the presence of ABT. The B-CMV2F1 cells overexpressing 2F1 demonstrated increased apoptosis (measured by Annexin-V binding) at 24 h with 100 μM 3MI. Therefore, CYP2F1 in human bronchial epithelial lung cells may bioactivate 3MI to 3-methyleneindolenine, which induces programmed cell death at relatively low concentrations. Human lung cells may be susceptible to this prototypical pneumotoxicant.

Key Words: 3-methylindole (3MI); human bronchial epithelial cells; BEAS-2B; cytochrome P4502F1 (CYP2F1); apoptosis.

3-Methylindole (3MI) is a species- and organ-selective pneumotoxin, showing its highest toxicity in goats and cattle, followed by rodents, while rabbits are relatively resistant to 3MI toxicity (Yost, 1999). The selective expression and catalytic activity of certain cytochrome P450 enzymes in lung cells are major factors in the pulmonary toxicity of several toxicants, including 3MI (Ramakanth et al., 1994; Yost, 1999). Humans are exposed to 3MI from colonic degradation of tryptophan and from cigarette smoke (Hoffman and Rathkamp, 1970), but the potential risk is unclear, because an appropriate in vitro cellular model of human susceptibility has not been established.

Isolated rabbit nonciliated bronchiolar epithelial cells (Clara) and type-II alveolar epithelial cells, which contain the highest concentrations of cytochrome P450 enzymes in the lung, were susceptible to cytotoxicity induced by 3MI (Nichols et al., 1990). Treatment with 1-aminobenzotriazole (ABT), a suicide inhibitor of CYP, protected these cells from 3MI-mediated toxicity, indicating a requirement for cytochrome P450 enzyme bioactivation. ABT has also been shown to inhibit 3MI turnover and covalent binding in human lung microsomes (Ruangyuttikarn et al., 1991). Isolated rabbit Clara cells efficiently bioactivated 3MI to form 3-methylenedione, which was detoxified by glutathione (GSH) (Thornton-Manning et al., 1993). These studies showed that the relatively low susceptibility of rabbits to the pneumotoxic effects of 3MI may be explained by the highly effective detoxication by nucleophilic trapping of the reactive 3-methylenedione with GSH, and is not due to less efficient formation of the reactive intermediate by P450 enzymes in rabbit Clara cells. One of the objectives in the current studies was to evaluate the hypothesis that human lung cells might also efficiently detoxify the reactive intermediate of 3MI though GSH conjugation.

Several studies have investigated which human P450 enzymes might be capable of bioactivating 3MI to an intermediate that covalently binds to cellular macromolecules. Although cytochrome P450s expressed in lung tissue are less well characterized than hepatic P450s, a cDNA encoding CYP2F1 was isolated from a human lung library (Nhamuro et al., 1990), and mRNA corresponding to this form was found in low abundance in several human lung samples. Human CYP2F1 bioactivated 3MI to a covalent-binding intermediate at a rate that was the highest among all human P450s tested by Thorn-
ton-Manning et al. (1991). In addition, CYP2F1 produced the highest rates of formation of 3-methyleneindolenine (Thorn-ton-Manning et al., 1996), the putative reactive intermediate, and CYP2F1 expressed in a lymphoblast cell line catalyzed only the dehydrogenation of 3MI without detectable formation of oxygenation products (Lanza et al., 1999; Lanza and Yost, 2001).

Some established human cell lines with indefinite life spans may serve as in vitro models to assess potential human toxicity. However, most of these cell lines have low or no metabolic capacity, primarily due to the loss of P450 enzyme expression. To overcome this limitation, metabolically competent human cell lines have been developed though cDNA overexpression techniques in order to restore specific catalytic activities (Macé et al., 1996). The human bronchial epithelial BEAS-2B cell line, immortalized by the SV-40 T-antigen gene (Reddel et al., 1988), retained the expression of several phase-II enzymes including glutathione S-transferase (GST), but expressed low levels of endogenous P450 (Macé et al., 1994). This cell line has been used to express recombinant CYP1A2 and was used for cytotoxic and genotoxic testing of aflatoxin B1 (Macé et al., 1997). Other human P450 enzymes that have been stably expressed in BEAS-2B cells include CYP2A6, 2B6, 2D6, 2E1, 3A4, and 3A5 (Macé et al., 1997).

In this study, BEAS-2B cells transfected with human cytochrome P450 cDNAs for 2A6, 3A4, 2E1, and 2F1 were used to evaluate the bioactivation of 3MI. Normal BEAS-2B cells were utilized to assess the unresolved molecular mechanisms by which electrophilic metabolites of 3MI induce cell death. The ability of 3MI to induce apoptosis was assessed in BEAS-2B cells by measuring the externalization of phosphatidylserine and by measuring DNA fragmentation. Preliminary studies using fluorescent microscopy after incubation of these cells with high concentrations of 3MI demonstrated apoptotic body formation as well as cell necrosis (Nichols et al., 2000). The current studies confirmed that P450-mediated bioactivation of 3MI induced DNA fragmentation and apoptosis in a human bronchial epithelial cell line and demonstrated that apoptosis occurred at low concentrations of 3MI. Overexpression of CYP2F1 increased the susceptibility of this human lung cell line to 3MI, as assessed by the extent of cytotoxicity and apoptosis.

**MATERIALS AND METHODS**

**Chemicals.** 3MI, DEM, ABT, glutathione, and DMSO were purchased from Sigma Chemical Company (St. Louis, MO). All other sources of chemicals and reagents were the highest purity available from local vendors, or their sources and are described within the individual methods sections.

**Human bronchial cell line.** BEAS-2B cells (American Type Culture Collection, Rockville, MD) were obtained by infection of normal bronchial epithelial cells with an adenovirus 12–simian virus 40 hybrid preparation (Reddel et al., 1988). To generate the CYP450-expressing vector, the 1.8-kb 2F1 (kindly provided by Frank Gonzalez, NIH, Bethesda, MD) cDNA was individually inserted by blunt-ended cloning into the BamH1 site of the pCVMneo vector (kindly provided by B. Vogelstein, Johns Hopkins University, Baltimore, MD). The DNA construct was introduced into the BEAS-2B cells by liposome-mediated transfection and selected as previously described (Macé et al., 1994). These immortalized clones are non-tumorigenic cells up to a very high passage number (Pfeifer et al., 1989). Control cells containing only the pCVMneo vector are designated B-CMVneo cells (Macé et al., 1997). The cells were cultured in a serum-free medium called LHC-9 (Biofluids, Rockville, MD). For subculturing, cells were trypsin-dissociated and plated into fibronectin/collagen-coated culture plates. The CYP450 expressing BEAS-2B cells utilized in the present study included three cell lines (B-CMV2A6, B-CMV3A4, B-CMV2E1) in which P450 expression has been previously characterized by western blot analysis and measurement of catalytic activities using specific fluorescent substrates such as 7-alkoxyxoumarins, coumarins, and 7-alkoxyresorufins (Macé et al., 1997). A non-toxic substrate for CYP2F1 is not available for quantitation of this enzyme’s activity.

**Western blot analysis of CYP2F1 in B-CMV2F1 cells.** Western blot analysis was done as described by Wang et al. (1998), using rabbit antipeptide polyclonal antibodies to CYP2F1 that were produced by Genemed Synthesis, Inc. (San Francisco, CA). These antibodies were raised to a keyhole limpet hemocyanin (KLH) conjugate of a cyclic amino acid peptide (KGCSCVH-DHQASLDPSPRDFIQC). This peptide was chosen to mimic the strategy employed by Schulz-Utermoehl et al. (2000). In this work, effective and selective antibodies were made to a hydrophilic loop region between helices G and I of CYP2F6. The peptide corresponding to residues 248–267 of CYP2F1 contained three extraneous amino acid N-terminal residues (KGC) designed to provide an N-terminal lysine to couple with KLH, a spacer glycine, and a cysteine that was oxidized with the other cysteine to form a disulfide to cyclize the peptide. These antibodies detected a 55 kDa protein band in the B-CMV2F1 cells that was not detected in the BEAS-2B cell line.

**Detection of CYP2F1 transcripts in B-CMV2F1 cells.** Total cellular RNA was isolated from confluent 75 cm² flasks using an RNeasy Kit and QIAshredder microspin homogenizer (Qiagen, Valencia, CA). RNA samples were stored at −70°C. First strand cDNA synthesis for RT/PCR was performed using Superscript II RNase H−reverse transcriptase ( Gibco BRL, Rockville, MD); 5 mg total RNA was used in each synthesis. The cDNA was stored at −20°C for PCR amplification.

PCR primers were designed to amplify a cytochrome P450 2F1 product, a small fragment of 427 bp that contained the entire CYP2F1 open reading frame. The small-fragment primers were 5’-GCTCGGAAAAACTGAAGG-3’ (sense) and 5’-GCCAAAGAGCAGGTTATGTGT-3’ (antisense). The PCR reactions were performed using 1 μl of cDNA (out of a 20-μl total from the reverse transcriptase step), 2.5 U Taq DNA polymerase (Gibco BRL, MD), 5 μl 10× PCR buffer, 1.5 μl 50 mM MgCl₂, 1 μl 10 mM dNTP mix (at a final concentration of 200 μM), 0.2 μM of each primer, and water to a final concentration of 50 μM. PCR reaction conditions were to denature at 94°C for 3 min, followed by 30 cycles of melting at 94°C for 1 min, annealing at 55°C for 1 min, extending at 72°C for 2 min, and adding a 10-min final extension. PCR product was visualized by electrophoresis on a 1% w/v agarose gel stained with ethidium bromide. The small fragment was cut from the gel and subcloned into pCR2.1-TOPO using the TOPO TA-cloning kit (Invitrogen, Carlsbad, CA) for sequencing.

**Lactate dehydrogenase leakage.** Cytotoxicity was measured by detection of the release of lactic dehydrogenase (LDH) into the media. Cultured cells were grown to confluence in 24-well plates coated with fibronectin and collagen in a humidified incubator with 95% air and 5% CO₂ in 0.5 ml of LHC-9 medium. 3MI and DEM were dissolved in DMSO, which was diluted to a final concentration of 0.5%. LDH leakage was determined by removing the supernatant from each well and centrifuging at 2000 × g for 10 min at 4°C. Total LDH was determined by adding 1% Triton X-100 to the replicate wells. LDH leakage and total LDH were determined using SIGMA Procedure No. 340-UV. The change in absorption/time was monitored every 11 s for a total of 6 min at 340 nm, using Molecular Devices SpectraMax 250 Microplate Reader (Molecular Devices, Sunnyvale, CA). Each experiment was carried out in triplicate and cytotoxicity was expressed in terms of cell viability deter-
mined by formula: 100 minus the percentage of total cellular LDH released into the medium. Cytotoxicity results were expressed as the percent of DMSO-treated control cell viability (86 ± 2%).

**Dojindo colorimetric cytotoxicity assay.** The cytotoxic effect of 3MI was assessed utilizing a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) (Dojindo Molecular Technologies, Inc., Gaithersburg, MD) bioassay, according to the manufacturer’s recommendation for this cell-counting kit. This bioassay utilizes Dojindo’s highly water-soluble tetrazolium salt, WST-8, which produces a water-soluble formazan dye upon reduction in the presence of an electron carrier. The amount of the formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells. Briefly, cells were subcultured into 96-well, collagen/bronectin-coated plates and grown to 50–80% confluence. A range of concentrations of 3MI in 0.5% v/v DMSO containing LH-9 serum-free medium, with or without 0.5 mM ABT, was subcultured into 96-well, collagen/fibronectin-coated plates and grown to confluence in Corning 150 cm² flasks on a collagen/fibronectin matrix. After 30 min. Finally, the microsomal pellet was resuspended in 0.25 M sucrose, containing LH-9 serum-free medium, with or without 0.5 mM ABT, was subcultured into 96-well, collagen/fibronectin-coated plates and grown to confluence in Corning 150 cm² flasks on a collagen/fibronectin matrix.

**Analysis of cellular glutathione content.** B-CMV2F1 cells were grown to confluence in 6-well plates on a collagen/bronectin matrix in a humidified atmosphere in 6-well plates on a collagen/fibronectin matrix in a humidified atmosphere in a humidified atmosphere in a humidified atmosphere in a humidified atmosphere in a humidified atmosphere. The confirmation that CYP2F1 was overexpressed in the B-CMV2F1 cell line.

**GST activity.** B-CMV2F1 cells were grown to confluence in Corning 150 cm² flasks on a collagen/bronectin matrix. After removing media, cells were trypsin-dissociated and centrifuged at 2000 × g for 7 min at 4°C to remove trypsin. The pellet (cells) was resuspended in a cold Tris–HCl buffer, pH 7.4 containing 10 mM Tris–HCl, 0.15 M KCl, 10 mM EDTA, and 1 mM dithiothreitol. Protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), was added to the 0.25 mM final concentration and cells were sonicated. Microsomes were prepared by centrifugation at a low speed (3000 × g for 5 min), followed by 10,000 × g for 10 min to remove the cell debris. Supernatant (S9 fraction) was then centrifuged at 105,000 × g for 30 min, and cytosol was separated. The pellet (microsomes) was washed three times with Tris–HCl buffer, resuspended, and recentrifuged at 105,000 × g for 30 min. Finally, the microsomal pellet was resuspended in 0.25 M sucrose, 0.05 M Tris, and 1 mM EDTA, pH 7.4, and stored at −80°C. Protein content of the cytosolic fraction was determined using the BCA protein assay.

**Glutathione S-Transferase (GST) activity.** B-CMV2F1 cells were grown to confluence in Corning 150 cm² flasks on a collagen/bronectin matrix. After removing media, cells were trypsin-dissociated and centrifuged at 2000 × g for 7 min at 4°C to remove trypsin. The pellet (cells) was resuspended in a cold Tris–HCl buffer, pH 7.4 containing 10 mM Tris–HCl, 0.15 M KCl, 10 mM EDTA, and 1 mM dithiothreitol. Protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), was added to the 0.25 mM final concentration and cells were sonicated. Microsomes were prepared by centrifugation at a low speed (3000 × g for 5 min), followed by 10,000 × g for 10 min to remove the cell debris. Supernatant (S9 fraction) was then centrifuged at 105,000 × g for 30 min, and cytosol was separated. The pellet (microsomes) was washed three times with Tris–HCl buffer, resuspended, and recentrifuged at 105,000 × g for 30 min. Finally, the microsomal pellet was resuspended in 0.25 M sucrose, 0.05 M Tris, and 1 mM EDTA, pH 7.4, and stored at −80°C. Protein content of the cytosolic fraction was determined using the BCA protein assay.

**GSH level.** GSH was measured with a modification of the method by Habig et al. (1974). Briefly, 15 mM GSH and 2.5 mM 1-chloro-2,4-dinitrobenzene (CDNB) were combined in a 1-ml cuvette in 0.1 M potassium phosphate buffer (pH 6.5) at room temperature.

**Annexin-V binding.** BEAS-2B cells were plated in LHC-9 media on FNC (FNC Coating Mix; Biological Research Facility and Facility, Inc.; Ijamsville, MD)-coated 6-well plates at 2 × 10⁵ cells per well, and the medium was replaced 48 h later. Five days after plating cells that had grown to subconfluence, the cells were treated for the times indicated and harvested with trypsin-EDTA and soybean trypsin inhibitor (SBI; BioFluids Division, BSI Rockville, MD). The detached cells were collected and added to the appropriate supernatant sample. Cells were centrifuged and then washed twice with cold DPBS + (with calcium and magnesium). After the last wash, the supernatant was aspirated and binding buffer (Annexin-V-FITC Apoptosis Detection Kit II, Pharmingen; San Jose, CA) was added to controls, Annexin-V-FITC only, propidium ioddide only, and block. The block control was incubated for 15 min at room temperature in the dark, then Annexin-V-FITC and propidium ioddide were added and the sample was incubated an additional 15 min. After all samples had been incubated for a total of 30 min, binding buffer was added and each sample was analyzed by flow cytometry on a FACScan flow cytometer (Becton Dickinson Company, Franklin Lakes, NJ).

For DMSO (dimethylsulfoxide) control cultures, forward-angle light scatter and side-angle light scatter were used to establish a gate of viable cells. The DMSO control samples were also used to set the regions of positive staining for Annexin-V-FITC and propidium ioddide. The Annexin-V-FITC–only controls and propidium ioddide-only controls were used to identify nonspecific binding. A four-quadrant dot plot was set was using these controls, and the percentage of cells reflect the number of events recorded in each quadrant.

**DNA fragmentation assay (cell-death assay for quantitative in vitro determination of cytoplasmic histone-associated DNA fragments).** Cells were incubated in 48-well plates (10⁴ cells per well) coated as described for Annexin-V studies. Experiments were initiated 24 h after plating cells that were at subconfluence when the test compounds were added. The adherent cells were gently lysed to release nucleosomes from the cytoplasm of apoptotic cells, using the buffer solutions provided in the commercially available Cell Death Detection ELISA kit (Boehringer-Mannheim, Indianapolis, IN) for the quantitative determination of in vitro cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes). The enrichment factor of nucleosomes in the cytoplasm of cells treated with 3MI compared to untreated cells was calculated using the formula: (absorbance at 405 nm using a reference absorbance at 490 nm for treated cells) divided by (corresponding absorbance for untreated control cells).

**Statistical analysis.** All the experiments were repeated, at least in triplicate, with three different passage numbers of cells. For a given set of experiments, each parameter, including controls, were evaluated with between 2 and 6 replicates, depending on the type of experiment. All the data were reported as mean ± SEM. The difference between (3MI) and (3MI + DEM) was tested using two-way analysis of variance (ANOVA). The individual groups were compared using Student’s t-test. The difference was considered significant with a probability of p ≤ 0.05.

**RESULTS**

**Overexpression of CYP2F1 in a human bronchial epithelial cell line.** The confirmation that CYP2F1 was overexpressed in the CMV-transfected BEAS-2B cell line (B-CMV2F1) was done using a 427-bp fragment of CYP2F1 for RT-PCR and western blot analysis, and using antipeptide antibodies to a fragment of CYP2F1. Total cellular RNA was isolated from control BEAS-2B, as well as from the B-CMV2F1 cell lines, and complementary DNA was produced with reverse transcriptase. PCR analysis, using the set of primers for the 427-bp fragment, produced only one detectable band in the agarose gel analysis at the appropriate mobility, which was only present in the B-CMV2F1 cell line (Fig. 1A). The large band was excised from the gel, subcloned into a TOPO TA-cloning kit, and submitted to the DNA sequencing facility at the University of Utah. The sequence of the large fragment corresponded exactly to the known genomic sequence of the CYP2F1 gene (Genbank Accession no. AC008962), originally cloned by Nhamburo et al., 1990). A low copy number of the 2F1 transcript was detected in BEAS-2B cells if a higher amount of cellular RNA was utilized for RT-PCR (data not shown). The western blot analysis for CYP2F1, using an antipeptide antibody to
CYP2F1, clearly showed only a 55 kDa band for the B-CMV2F1 cell line that was not detectable in the control BEAS-2B cells (Fig. 1B).

Role of specific human CYPs in 3MI cytotoxicity. The bioactivation of 3MI to a cytotoxic intermediate was assessed by measuring the leakage of LDH into the medium by the human bronchial epithelial cell line, BEAS-2B, and four different CMV transfected BEAS-2B lines that express the product of human cDNAs for P450s 2A6, 3A4, 2E1, and 2F1; B-CMV2A6, B-CMV3A4, B-CMV2E1, and B-CMV2F1, respectively.

The CMV transfected cell lines were incubated with 1 mM 3MI for 48 h in the presence and absence of 5 mM ABT, a suicide substrate inhibitor of cytochrome P450. Cytotoxicity was measured by the amount of LDH released into the medium compared to that by control untreated cell cultures (Fig. 2). The B-CMV2F1 cells were the most susceptible to 3MI-mediated cytotoxicity after 48 h, followed by B-CMV3A4 (34 and 45% viability of control, respectively). BEAS-2B and B-CMVneo were only slightly affected (80% viability of control) by 3MI under the same conditions. The responses of B-CMV2A6 and B-CMV2E1 cells were not significantly different from control cells. The presence of ABT protected the susceptible cells from 3MI-mediated cytotoxicity, indicating a requirement for P450-mediated bioactivation of 3MI.

Changes in 3MI cytotoxicity with glutathione depletion. A time course of 3MI cytotoxicity in B-CMV2F1 cells is shown in Figure 3. The earliest significant decrease in cell viability with 1 mM 3MI was observed at 48 h. The depletion of glutathione stores by 0.5 mM diethylmaleate (DEM) did not significantly decrease cell viability, even at 48 h (93%, data not shown). However, the combination of DEM with 3MI caused cytotoxicity at 18, 22, and 24 h, whereas 3MI alone did not show cytotoxicity at these times. The cell viability after treatment with both 3MI and DEM was decreased to 65 and 54% of the untreated control cells at 18 and 24 h, respectively. The combination of 3MI with DEM was also more toxic at 48 h (24% of control) than that observed with 3MI alone (46% of the control). The results of this time course demonstrated that DEM treatment potentiated the cytotoxic effects of 3MI in B-CMV2F1 cells.

The role of glutathione depletion in 3MI-mediated cytotoxicity was assessed by measuring total GSH levels in the treated cells. The GSH levels in B-CMV2F1 cells incubated with 3MI (0.75 and 1 mM) for various time periods from 5 min to 24 h were not different from controls (data not shown). Early time periods (5, 10, 20, 30, and 60 min) as well as longer times (4, 8, 12, 16, and 24 h) were examined. DEM (1 mM), alone and in combination with 1 mM 3MI, decreased GSH levels to 72 and 65% of untreated control cells (7.6 ± 1.3 nmol/10^6 cells), respectively. In the presence of 3MI, however, the depletion was not enhanced, reinforcing the results that 3MI did not decrease GSH levels in these cells.

A role for GST in the detoxification of 3MI was suspected, based on the increased susceptibility of cells treated with
DEM, which decreased GSH levels without causing cytotoxicity. Therefore, the GST activity in the cytosol of B-CMV2F1 cells was measured at different protein concentrations. The GST activity was linear in the range of 0.1–0.4 mg protein with the correlation coefficient of 0.96. The GST activity in the cell cytosol was 101 ± 21 nmol/min/mg protein with 15 mM GSH and 2.5 mM CDNB. The experiment was carried out at room temperature, and the value is a mean of 3 different batches of cell cytosol, each with a different passage number of cells. The activity was measured in duplicate for each individual batch of cell cytosol. The reported activity is the mean ± SEM. The GST activity in microsomes was undetectable with these conditions.

Increased susceptibility of B-CMV2F1 cell line to low concentrations of 3MI. The relative susceptibility of the B-CMV2F1 and BEAS-2B cell lines to 3MI-induced cytotoxicity was assessed over a range of concentrations from 5 to 100 μM by measuring the metabolic activity of the cells, using the Dojindo bioassay for cell viability. The results shown in Figure 4 clearly demonstrated that B-CMV2F1 cells were more susceptible to 3MI-induced cytotoxicity after a 24-h incubation at 10 μM 3MI than were the BEAS-2B control cells (10% vs. 50%, respectively). The increased susceptibility of the 2F1 overexpressing cells compared to BEAS-2B cells was also statistically significant at 50 and 100 μM 3MI (5% vs. 20%, respectively).

Apoptosis in BEAS-2B cells subsequent to bioactivation of 3MI. Normal BEAS-2B cells (without overexpression of P450s) were incubated in 35-mm culture plates with a high concentration (1 mM) of 3MI for various times from 12 to 48 h, to assess whether the mechanism of cytotoxicity was associated with cell necrosis or apoptosis. DNA fragmentation was studied by quantitative in vitro ELISA, which measured the relative enrichment of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) in BEAS-2B cells incubated with 1 mM 3MI for 12 to 48 h. 3MI increased DNA fragmentation at 12 h, and progressively increased it at 24, 36, and 48 h compared to untreated control cells (data not shown). A dose-response study of the apoptotic changes induced by 3MI was done at 24 h (Fig. 5). Low concentrations of 3MI (10–100 μM) induced DNA fragmentation that was dose-dependent and was inhibited by the P450 inhibitor, ABT. These experiments demonstrated that normal BEAS-2B cells express P450 enzymes that bioactivate low concentrations of 3MI to a reactive intermediate that induces apoptosis.

Early apoptotic changes induced by 3MI in BEAS-2B cells. We measured the ability of 3MI to increase Annexin-V binding to BEAS-2B cells by using FACS to assess externalization of phosphatidylserine on the cytoplasmic membrane of cells, an early event detected in apoptotic cells. A significant increase in Annexin-V binding was detected as early as 6 h in BEAS-2B cells incubated with 50 and 100 μM 3MI (Fig. 6). Only intact
BEAS-2B cells were counted in these experiments, because any leaky cells that stained positive with propidium iodide were excluded.

**Increased apoptosis induced by 3MI in BCMV2F1 cells.** The extent of apoptosis induced by 10 and 100 μM 3MI was compared for B-CMV2F1 and BEAS-2B control cells by measuring the percentage of Annexin-V-positive viable cells using FACS (Fig. 7). The 2F1 overexpressing cells clearly demonstrated an increased percentage of Annexin-V-positive cells compared with control BEAS-2B cells after a 24-h incubation with 100 μM 3MI (43% vs. 27%, respectively). The presence of ABT inhibited bioactivation of 3MI and decreased apoptosis in both B-CMV2F1 and BEAS-2B cells.

**DISCUSSION**

CYP2F1 has been firmly established as an efficient catalyst of 3MI dehydrogenation (Lanza et al., 1999; Lanza and Yost, 2001; Thornton-Manning et al., 1996), but the role of this bioactivation process in a human bronchial epithelial cell line overexpressing this specific human pulmonary P450 enzyme was not established prior to the current studies. Among all the cell lines tested, B-CMV2F1 clearly showed the highest susceptibility to 3MI, and the toxicity was essentially reversed by pretreatment with ABT, a suicide substrate inhibitor for this cytochrome P450s. The low level of expression of cytochrome P450s in BEAS-2B cells (Macé et al., 1998) explains the low susceptibility to 3MI observed with the BEAS-2B and B-CMVneo control cells. Human bronchial mucosal cells have been shown to express several cytochrome P450s, including 1A1, 2C8, 2C18, 2A6, 2B6, 2E1, 2F1, and 3A5 (Anttila et al., 1997; Willey et al., 1997). Higher levels of 1A1 and 1B1 are observed in human bronchial epithelial cells of smokers (Willey et al., 1997). Although the bioactivation of 3MI in human lung cells may involve more than one cytochrome P450 enzyme, the current studies support the hypothesis that CYP2F1-mediated dehydrogenation of 3MI is a potential pathway that may induce bronchial epithelial-cell injury in normal human lung cells that express higher levels of this enzyme.

Cytotoxicity of bioactivated compounds depends upon the balance between activation and the ability of the cells to detoxify the resulting electrophilic metabolites. In general, metabolites generated by cytochrome P450 enzymes cause cytotoxicity by a combination of processes that can involve direct interaction with critical macromolecules in target cells and/or depletion of cofactors important in detoxification processes such as GSH, which may indirectly result in cell injury. Studies by Nocerini et al. (1983), exemplifying these processes, have demonstrated that 3MI depletes GSH significantly in lung, liver, and kidney tissues when administered to goats. Depletion of pulmonary GSH by administration of DEM prior to 3MI administration significantly enhanced pneumotoxicity in goats. Conversely, administration of a GSH precursor, cysteine, a rate-limiting compound in cellular GSH biosynthesis, prolonged survival times, decreased covalent binding of 3MI metabolites, and reduced the severity of 3MI-induced lung injury when given prior to 3MI administration (Nocerini et al., 1983).

Studies with several animal species have been reported which document that 3MI induces severe pulmonary damage to epithelial cells in the bronchioles and alveoli after systemic administration of this toxicant (Adams et al., 1988; Yost, 1999). In order to study the involvement of GSH in a human lung cell model, B-CMV2F1 cells were incubated with 3MI in the presence of DEM, and 3MI toxicity was apparent after a shorter time of incubation (18 vs. 48 h, Fig. 3). However, the GSH levels were not decreased by 3MI alone at any of the times studied, from 5 min to 48 h. The human B-CMV2F1 cells were more susceptible to 3MI if the availability of glutathione was reduced by DEM, but depletion of total cellular GSH only increased susceptibility by about 50%. These results can be contrasted with previous studies with isolated rabbit Clara cells (Thornton-Manning et al., 1993), which showed that GSH adduct formation efficiently detoxified 3MI reactive intermediates to protect rabbits from 3MI-mediated pneumotoxicity. The cytosolic fraction of the B-CMV2F1 cells showed a GST activity (101 ± 21 nmol/min/mg protein) that is in close agreement with the published value of GST activity reported for BEAS-2B cells (91.9 nmol/min/mg protein) by Macé et al. (1997) and for human lung cells (77 nmol/min/mg protein) by Tateoka et al., 1987. In the current study, B-CMV2F1 cells were shown to possess relatively normal levels of GSH and active GST enzymes, but detoxification via this pathway did not efficiently protect these human lung cells from 3MI-mediated damage.

High concentrations of 3MI clearly induced cell necrosis based on the measurement of leakage of lactate dehydrogenase into the medium in these human lung-cell lines. However, it
was also evident that bioactivation of 3MI by BEAS-2B cells can lead to a dose-dependent induction of apoptosis when low concentrations (10–100 μM) of this selective pneumotoxicant are present. Inhibition of the P450 enzymes in the BEAS-2B cells protected cells from both necrosis and apoptosis at early time periods (12–24 h). The increased extent of cytotoxicity and apoptosis observed in the lung-cell-line overexpression of CYP2F1 demonstrated a clear role for this pulmonary-expressed enzyme in the bioactivation of 3MI to a cytotoxic intermediate.

The ability of CYP2F1 to metabolize 3MI to a metabolite that can alkylate DNA through the covalent bond formation of 3-methyleneindolenine with the exocyclic amine nitrogens of deoxyguanosine and deoxycytidine has recently been demonstrated (Regal et al., 2001). These adducts were formed from in vitro microsomal incubations with calf thymus DNA, as well as with intact DNA from incubations of 3MI with rat hepatocytes. Therefore, apoptosis caused by 3MI could occur through the direct alkylation of nuclear DNA, which signals cellular apoptotic mechanisms in BEAS-2B cells.

The ability of a low concentration of 3MI to induce apoptotic changes in BEAS-2B cells as early as 6 h was clearly demonstrated using Annexin-V binding, which occurred in cells that were not necrotic. The earliest evidence of necrosis, measured in 2FI overexpressing cells treated with DEM to deplete glutathione, was at 18 h when cells were incubated with a high concentration of 3MI. Consequently, the apoptotic pathway was initiated at a much lower concentration of 3MI than the concentration required to induce cellular necrosis. A conclusion consistent with these results is that 3MI causes cell death through an apoptotic mechanism, even at relatively low concentrations and at shorter time periods than observed with studies that measured leakage of LDH into the media. However, when higher concentrations and/or longer time periods are evaluated, the cells become “leaky” and secondary necrosis can be observed.

In conclusion, BEAS-2B cells transfected with CYP2F1 and CYP3A4 were susceptible to 3MI-mediated cytotoxicity, which was moderately enhanced if cells overexpressing CYP2F1 were treated with DEM, a chemical known to deplete cellular GSH. Studies of 3MI metabolism by recombinant CYP3A4 showed that only 3-methylindole was produced. However, reactive intermediates (the 2,3-epoxide and 3-hydroxy-3-methylindolenine) may be formed from the ring oxidation of 3MI, as reported in earlier studies by our laboratory (Skordos et al., 1998a,b). Recombinant CYP2F1 only produced the dehydrogenation product of 3MI (Lanza and Yost, 2001), which covalently bound to cellular macromolecules (Thornton-Manning et al., 1991). The dehydrogenated metabolite, 3-methyleindolenine, was the only 3MI product detected from human HepG2 cells infected with vaccinia virus containing CYP2F1 cDNA (Thornton-Manning et al., 1991). Production of the dehydrogenated metabolite by various P450 enzymes in this cellular model was precisely correlated to the relative levels of covalent binding to cellular proteins. Therefore, the current result with B-CMV2F1 cellscorrelated very well with previous studies from our laboratory that CYP2F1 efficiently produces 3-methyleindolenine, which covalently binds to critical cellular proteins or DNA (Regal et al., 2001) and results in cell death. It was apparent from the current studies that human P450 enzymes participate in the bioactivation of 3MI to reactive intermediates that initiate apoptosis in human lung cell lines. Since the BEAS-2B cell line was derived from normal human bronchial epithelial cells, these results strongly suggest that human lung epithelial cells that express higher levels of this enzyme may be susceptible to damage by 3MI, which may induce DNA fragmentation and apoptosis.

ACKNOWLEDGMENTS

This work was supported by Grant HL13645 from the National Heart, Lung, and Blood Institute of the National Institutes of Health. We are grateful to Huifen Wang, Diane Lanza, and Fredine Lauer for their technical assistance in completing the western immunoblots and the FACS analysis.

REFERENCES


