Expression of Xenobiotic-Metabolizing Enzymes in Cultured Rat Tracheal Epithelial Cells

André Castonguay,1 Lila Overby, Paul Nettesheim,2 George C. Clark,2 and Richard M. Philipot2

1School of Pharmacy, Laval University, Quebec City, GIK 7K4 Canada; 2National Institute of Environmental Health Sciences, Research Triangle Park, NC 27706 USA

Rat tracheal epithelial (RTE) cells were cultured on membrane support with and without retinoic acid (RA). In early (6-day-old) cultures, the epithelium is a monolayer or bilayer of undifferentiated cells and secretes little mucouslike product either in the absence or presence of RA. In late (12- to 15-day-old) cultures, the epithelium differentiates as a mucociliary epithelium in the presence of RA and as a squamous epithelium in the absence of RA. The purpose of our study was to determine whether a number of xenobiotic enzymes are expressed in these cultures and whether their expression depends on the state of differentiation. Enzyme expression was characterized by electrophoresis and immunoblotting as a function of time in culture and phenotypic differentiation. Cytochrome P450 1A1 was not expressed in freshly harvested RTE cells. This isoenzyme was induced in rats by gavage with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or by exposure of early RTE cell cultures to TCDD, provided RA was also added to the cultures. Cytochrome P450 2B1 was observed in freshly isolated RTE cells, but not in early or late RTE cultures. In contrast, expression of NADPH-cytochrome P450 reductase was decreased in early cultures, but was increased in well-differentiated cultures. Flavin-containing monoxygenase was detected in lung tissue, but not in freshly harvested or cultured RTE cells. Glutathione S-transferases (GST) ι and π were expressed in freshly harvested RTE cells. GST π was expressed in early and late cultures, whereas GST ι was expressed in late cultures, but could not be found in early cultured RTE cells. Levels of GST isoenzymes were unaffected by RA. These results parallel the expression of enzymes observed in proliferating and differentiating epithelium induced by mechanical injury in vivo. Induction of monoxygenases by polycyclic aromatic hydrocarbons results in RTE cells with metabolic activating and deactivating enzymes and constitutes a system suited for some toxicological studies. Key words: cytochrome P450; flavin-containing monoxygenase, glutathione transferase; mucin; rat tracheal cells; retinoic acid; TCDD. Environ Health Perspect 103: 254–258 (1995)

The carcinogenic potential of chemicals in a given tissue may be determined by the ability of the tissue to activate and detoxify these chemicals. Most chemical carcinogens are activated by the cytochrome P450 monoxygenase system, which comprises the hemoprotein cytochrome-g and the flavoprotein NADPH-cytochrome P450 reductase. The cytochromes P450 are a superfamily of enzymes (1); subfamily P450 1A catalyzes the activation of many carcinogens, including benzo[a]pyrene, aflatoxin B1, and tobacco-specific nitrosamines (2). A second monoxygenase system, the flavin-containing monooxygenase (FMO), is involved in the activation of aromatic amines and in the detoxification of carcinogens having a pyridine ring (3,4). Glutathione S-transferases (GSTs) are an important class of carcinogen-detoxifying enzymes that catalyze the nucleophilic attack of the thiol group of glutathione on reactive electrophilic metabolites, including proximate and ultimate carcinogens (5). Expression of these enzymes is usually down-regulated in human and animal tissues cultured in vitro.

Maintenance of enzyme expression in cultured cells depends on culture conditions, cell type, and the enzyme considered. For instance, under conventional culture conditions, rat hepatocytes lose 80% of their cytochrome P450 during 24 hr of culture and, during the same period, lose 50% of their NADPH-P450 reductase activity (6). Recently, Schuetz et al. (7) developed a membrane matrix, “matrigel,” which allows for the in vitro induction of some cytochrome P450 isoforms to levels observed in vivo. Under these conditions, secretions of proteins considered markers of hepatocyte differentiation increased significantly in cultures, whereas α-fetoprotein, an indicator of dedifferentiation, was not observed (7). These studies demonstrated that maintenance of in vitro-like cellular morphology correlates with the preservation of activities and expression of enzymes in cultured cells.

The normal pseudostratified columnar epithelium of rat trachea consists of basal, secretory, and ciliated cells. Considerable efforts by various investigators have been directed toward maintaining the differentiated morphology of tracheobronchial cells in vitro (8–11). Trump et al. (12) hypothesized that tracheobronchial mucous cells are the major target for initiation of tumorigenesis. Recently, we used a biphasic culture system to obtain mucociliary differentiation of RTE cells in the presence of retinoic acid (RA). In biphasic cultures, a mucus phenotype is induced by RA. With no RA in the culture medium, a metaplastic stratified squamous epithelium develops (13).

Several enzymes involved in drug metabolism can be induced in the lung by exposure to environmental chemicals. One potent inducer of several xenobiotic-metabolizing enzymes is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The mechanism for TCDD induction of these enzymes is fairly well understood, at least for P450 1A1. TCDD binds to a specific high-affinity cytoplasmic receptor (the Ah receptor); the receptor-TCDD complex then translocates to the nucleus and binds to upstream regulatory elements, resulting in transcriptional activation of the CYP1A1 gene (14,15). Other genes forming the Ah gene battery are thought to be similarly induced to high level expression in sensitive tissues (16). It is important to determine whether these induction processes are functional in cultures of airway cells.

The aim of this study was to determine the expression of various enzymes important in chemical toxification/detoxification in RTE cells cultured under newly developed conditions. Expression of these enzymes was compared in RTE cells differentiated into stratified squamous or pseudostratified mucociliary epithelium.

Materials and Methods

Male F344 rats were bred in the National Institute of Environmental Health Sciences colony. A stock solution of TCDD in corn oil (50 μg/ml; lot 2715-020-016-CO; purity >98% by gas chromatography/mass spectrometry) was purchased from Radian Corporation (Austin, Texas). This solution had been prepared by diluting TCDD into an acetone:corn oil emulsion and removing the acetone by rotary evaporation. Rats were treated once with 50 μg TCDD/kg (0.1 ml/100 g body weight, per os). The rats were euthanized with CO2 7 days after exposure to TCDD, and tracheal epithelium was isolated.

Tracheas were cannulated, and tracheal
epithelial cells were isolated by pronase digestion as described previously (13). The RTE cells were seeded at a density of 1.2 × 10^5 cells/insert and cultured on Transwell-Col tissue culture inserts (24.5 mm diameter; Costar Co., Cambridge, Massachusetts). Cells were cultured with or without 10 nM RA as described previously (13).

We dissolved TCDD (lot MLB 15091-55) purchased from Cambridge Isotope Laboratories, Inc. (Woburn, Massachusetts) was dissolved in toluene (1 mg/ml) and serially diluted in dimethyl sulfoxide (DMSO) to a concentration of 10 µM. Just before medium change, aliquots of this solution were added to the culture medium to a final dilution of 10 nM TCDD.

We removed RTE cells from the inserts by treatment with 0.25% trypsin-EDTA (0.3 ml/insert) at 37°C for 2 min. After adding 0.5 ml phosphate-buffered saline (PBS) containing aprotinin (50 µg/ml), leupeptin (10 µg/ml), and phenylmethylsulfonyl fluoride (44 µg/ml), the cell suspension was removed by suction from the insert. We washed each insert three times with PBS containing the inhibitors described above. The cell suspension was centrifuged at 1000g for 10 min. The pellet was homogenized in a Broek tissue grinder and sonicated three times at 4°C. After centrifugation (900g, 0°C, 10 min) the supernatant was fractionated at 110,000g for 1 hr at 4°C. The microsomal fraction was dispersed in 1 mM EDTA, 10 mM Tris HCl, and 20% glycerol buffer. Protein concentrations were determined with bicinechonic acid/copper sulfate reagents using bovine serum albumin as standard (Pierce; Rockford, Illinois).

Culture medium (0.5 ml) present in the inserts on day 6 of culture was removed after a period of 24 hr (day 7 of culture). Each insert was washed twice with 0.5 ml of PBS. On day 12, we washed inserts with 0.5 ml PBS. Culture media and washings were combined, and aliquots were diluted 10-fold (day 6) or 200- to 2000-fold (day 12) with 10 mM EDTA and 0.6 M KSCN. Aliquots were applied to nitrocellulose paper using a slot-blot apparatus (Schleicher & Schuell, Keene, New Hampshire). The nitrocellulose was treated successively with 1) 5% nonfat milk in PBS containing 0.05% Tween 20 for 30 min; 2) monoclonal antibody RTE 11 against mucuslike secretions diluted 1:20 for 30 min; 3) peroxidase conjugate donkey anti-mouse IgG (Jackson Immunoresearch, Philadelphia, Pennsylvania) diluted 1:3000; and 4) 10 µg 3,3'-diaminobenzidine, 100 µl of 3% H2O2 in 100 ml of 0.05 M Tris HCl buffer, pH 7.6. Intensities of the bands were quantitated with a video densitometer (model 620; Bio-Rad, Rockville Centre, New York) in the reflection mode.

Electrophoresis of microsomal and cytosolic proteins was carried out on 7.5% and 10% polyacrylamide gels, respectively, in the presence of sodium dodecyl sulfate (SDS). Samples were applied to 5x Laemmli buffer (0.312 M Tris, pH 6.8; 0.6% β-mercaptoethanol; 10% sucrose; 1% SDS). Cytosolic samples were heated at 95°C for 3 min. Transfer to nitrocellulose and immunostaining using rabbit anti-goat IgG (dilution 1/100) and goat peroxidase-immunoperoxidase (Organon Teknika Corp., Westchester, Pennsylvania) have been described (17,18). The specificities of the following goat antibodies have been described previously: goat anti-rat P450 1A1 (19); goat anti-rat P450 2B (20); goat anti-rabbit NADPH P450 reductase (21); goat anti-pig liver FMO 1A (18); and goat anti-rabbit GST μ and goat anti-rabbit GST π (22).

Results

RTE cells, cultured for 6 days on transwell inserts in the presence or absence of RA (Fig. 1), formed a flat monolayer of undifferentiated epithelial cells. On day 12, the cultures supplemented with RA showed a pseudostratified epithelium containing many alcin blue-phosphatase alkaline substrate-positive secretory cells (Fig. 1B). Ciliated cells only appeared between days 14 and 17 (not shown). At day 12, the RA-deprived cultures showed a thick, keratinizing squamous epithelium (Fig. 1D).

The monoclonal antibody RTE 11, specific for granule components of secretory cells, was used to assay for the production of mucinlike glycoproteins by cultured RTE cells (23). As shown in Figure 2, RTE cells cultured for 6 days produced only low levels of mucinlike material. On day 12 of culture, the amount of RTE-11 reactive product increased. RA-treated cultures produced more than 10-fold greater amounts of mucinlike material than RA-deficient cultures.

Analysis of P450 1A1 by immunoblotting showed that freshly harvested RTE cells do not express this P450 isoform (Fig. 3A) and that P450 1A1 could not be detected in lungs of untreated rats. However, P450 1A1 was induced in RTE cells and in lung by treatment of rats with TCDD 1 week before sacrifice. RTE cells cultured for 13 days and subsequently exposed to TCDD for 2 days did not express P450 1A1 (Fig. 3A, lanes 6 versus 5). P450 1A1 was also detected in samples of TCDD-induced rat liver.

Figure 1. Representative photomicrographs of rat tracheal epithelial cells cultured on Transwell inserts, cut in 2-µm-thick plastic sections, and stained with alcian blue-periodic acid–Schiff-hematoxylin. (A) Day 6, plus 10 nM retinoic acid (RA); (B) day 6 minus RA; (C) day 12 plus 10 nM RA; (D) day 12 minus RA. On day 6, both with and without RA, the cells appear flat and undifferentiated. On day 12 in cultures containing RA, the epithelium is pseudostratified columnar and contains numerous secretory cells (arrowheads). On day 12 in cultures without RA, the epithelium is stratified squamous and exhibits epidermoid differentiation. Bar = 50 µm.
As shown in Figure 3B, expression of CYP1A1 was induced by exposing early RTE cultures to TCDD. P450 1A1 was induced by TCDD only in RTE cells exposed to RA and differentiating into the mucociliary phenotype (Fig. 3B, lanes 5 versus 6). P450 1A1 was not expressed in RTE cultured for 16 days with or without RA (Fig. 3B; Table 1). P450 1A1 was detected in livers of TCDD-treated rats, but could not be detected in rat lungs at a concentration of 50 μg of microsomal protein.

Although P450 2B1 was present in untreated rat lungs (Fig. 4) and in freshly harvested RTE cells, it was not detected in early RTE cultures (Fig. 4; Table 1). This isoform was also not detected in late RTE cells cultures differentiated as squamous (without RA) or mucociliary (with RA) epithelium (data not shown).

Figure 5 shows the expression of NADPH-cytochrome P450 reductase in RTE cells. This enzyme was expressed in freshly harvested RTE cells. This expression decreased after shorter times in culture but was higher after 14 days in culture. The presence of RA in the culture medium had no apparent effect on the expression of this enzyme either after 6 days (Fig. 5, lanes 6 versus 7) or 14 days in culture (Fig. 5, lanes 11 versus 12; Table 1). This enzyme was detected in samples from rat liver, rabbit liver, and in rat lung.

As shown in Figure 6, a single FMO-1 band was detected in microsomes from rat lung and pig liver. In contrast, this enzyme was not detected in freshly harvested RTE cells or RTE cells cultured for 15 days.

A GST μ isoform was expressed in freshly harvested RTE cells but was not detected in RTE cells cultured for 6 days (Fig. 7; Table 1). GST μ was expressed in RTE cells cultured for 15 days. Culturing RTE cells with RA had no effect on the expression of this GST (Fig. 7, lanes 3 versus 4, 7 versus 8). This enzyme was expressed in rabbit liver, rat liver, and in the lung of TCDD-treated rats.

The expression of the GST isoform π is shown in Figure 8. GST π was expressed in freshly harvested RTE cells, in RTE cells cultured for 6 days, and in RTE cells cultured for 15 days. RA had no effect on

### Table 1. Expression of xenobiotic-metabolizing enzymes in cultured RTE cells

| Days in culture | RA(*) Inducers | P450 1A1 | P450 2B1 | Reductase | FMO | GST μ | GST π |
|----------------|----------------|----------|----------|-----------|-----|-------|-------|
| 0              | None           | –        | –        | +         | –   | +     | +     |
| 6              | None           | –        | –        | +         | –   | ND    | ND    |
| 13–16          | None           | –        | –        | +         | –   | ND    | ND    |
| 16             | None           | –        | –        | –         | +   | ND    | ND    |
| 13–16          | TCDD           | +        | –        | +         | ND  | ND    | ND    |
| 16             | TCDD           | +        | ND       | ND        | ND  | ND    | ND    |

Abbreviations: RA, retinoic acid; FMO, flavin-containing monooxygenase; GST, glutathione-S-transferase; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; ND, not determined.

(*) RA, 10 nM.
Expression of enzymes in cultured RTE cells

In this study, we demonstrated that tracheal expression of P450 1A1 can be induced by a single treatment of rats with TCDD (Fig. 3A). In vitro exposure of RTE to TCDD did not induce P450 1A1 unless retinoic acid was included in the culture system of proliferating cells (Fig. 3B). TCDD induction of P450 1A1 was observed in undifferentiated proliferating RTE culture but not in growth-arrested, well-differentiated culture (Fig. 3). This is consistent with the results of Clark et al. (29), who observed that human peripheral blood lymphocytes do not express the Ah receptor until the cells have been stimulated to proliferate.

Altered morphology, kinetics of cell regeneration, and enzyme expression following mechanical injuries have been documented in rat and hamster tracheas. After mild abrasion of the rat tracheal epithelium, basal cells remaining at the site of injury undergo flattening in order to re-form a complete cell layer. This is followed by increased mitotic activity (25). In hamsters, this single-layer epithelium changes rapidly to a multilayered epithelium. In the final stage of healing, regenerating cells showing early stages of ciliogenesis and formation of mucous granules are observed (26). The insert culture of RTE cells described in this paper and in a previous report from this laboratory (13) exhibits the same sequence of proliferation and differentiation observed in vivo. During regeneration of the epithelium in hamsters, the expression of a P450 2B isozyme is reduced significantly. This isoform is strongly expressed in secretory cells of intact epithelium. In line with this observation, we did not observe P450 2B1 expression in proliferating RTE cultures (Fig. 4). Similarly, McDowell et al. (27) did not observe cytochrome P450 reductase expression in regenerating tracheal epithelium. In our study, the expression of cytochrome P450 reductase was lower in proliferating RTE cells than in freshly harvested RTE cells or well-differentiated RTE cells (Fig. 5). Taken together, our data show that cultures of RTE cells on membrane support with an air-liquid interface are not only morphologically but also biochemically comparable to tracheal regeneration in vivo.

The GSTs are a family of enzymes involved in the detoxification of electrophilic intermediates generated during the metabolism of toxic agents. They catalyze the conjugation of electrophilic intermediates to the tripeptide glutathione (GSH). The rat enzymes are divided into three classes, α, μ, and π, according to their structures and enzymatic properties (28). Their levels of expression differ from tissue to tissue and within different cells of the same tissue (29). The expression of α and μ transferases are low in rat lung (30). In this study, we demonstrated that GST μ is expressed in freshly harvested RTE cells and in RTE cells differentiated into squamous or mucociliary phenotype, but not in proliferating RTE cells (Fig. 7).

Results from this study and from previous studies show that RTE cells express various markers of squamous or mucociliary differentiation after 6 days in culture (13). Preliminary results from this laboratory showed that RTE cells seeded at 1.2 × 10⁶/insert were in proliferation during the first 6 days of culture. At this stage of culture, RA has little effect on cell morphology (unpublished results). Culturing RTE cells for 12–15 days, with or without RA, induced a mucociliary or squamous differentiation, respectively. GST π was expressed in RTE cells having either phenotype as well as in proliferating RTE cells, demonstrating that GST π would be available to detoxify carcinogen-derived electrophilic species that are substrates for this enzyme, regardless of the proliferative or differentiated stage of RTE cultures.

In summary, biphasic RTE cell culture provides a versatile system that shows excellent potential for screening environmental carcinogens and chemopreventive agents involved in the detoxification of carcinogen derived intermediates.

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