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Differentiation-Dependent Induction of *CYP1A1* in Cultured Rat Small Intestinal Epithelial Cells, Colonocytes, and Human Colon Carcinoma Cells: Basement Membrane-Mediated Apoptosis

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Abstract Rat small intestinal epithelial cells and human colon adenocarcinoma cells cultured on Matrigel™ expressed the differentiation specific enzyme, sucrase-isomaltase, as determined by indirect immunofluorescence. Rat small intestinal epithelial cells, rat colonocytes, and human colon adenocarcinoma cells developed an altered morphology when cultured on Matrigel and became apoptotic within 24–48 h. Benzo[*a*]pyrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin caused a 2- and 5-fold induction, respectively, of ethoxyresorufin-*o*-deethylase activity in rat small intestinal epithelial cells cultured on Matrigel. Benzo[*a*]pyrene- or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced ethoxyresorufin-*o*-deethylase activity in rat small intestinal epithelial cells cultured on plastic was not detected. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin treatment caused a 14-fold induction of transfected, rat *CYP1A1*-promoter-luciferase activity in rat small intestinal epithelial cells cultured on Matrigel. Benzo[*a*]pyrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin treatment induced ethoxyresorufin-*o*-deethylase activity by 6- and 1.6-fold, respectively in rat colonocytes cultured on Matrigel. Induction of ethoxyresorufin-*o*-deethylase activity was not observed in rat colonocytes cultured on plastic. *CYP1A1*-promoter-luciferase activity was induced 3-fold by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rat colonocytes cultured on Matrigel. Induction of *CYP1A1*-promoter-luciferase activity in rat small intestinal epithelial cells or rat colonocytes cultured on plastic was not observed. Ethoxyresorufin-*o*-deethylase activity in human colon adenocarcinoma cells, cultured on either plastic or Matrigel, was induced 7-fold by benzo[*a*]pyrene. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced ethoxyresorufin-*o*-deethylase activity was 2-fold greater in human colon adenocarcinoma cells cultured on Matrigel compared to cells cultured on plastic. Extracellular matrix-mediated differentiation and apoptosis of intestinal cells provide in vitro systems for study of the regulation of *CYP1A1* expression, carcinogen activation in the gut and mechanism(s) of apoptosis of colon cancer cells. J. Cell. Biochem. 86: 440–450, 2002.

Key words: CaCo-2; IEC-6; IEC-17; B[*a*]P; TCDD; dioxin; polycyclic aromatic hydrocarbon; dietary carcinogens

The cytochrome P450 superfamily of proteins are involved in the biotransformation of a number of endogenous and exogenous substances including steroids, certain fatty acids, chemotherapeutic agents, pesticides, and environmental contaminants [Gillette et al., 1972; Conney, 1982; Nebert et al., 1989]. The cytochrome P4501 subfamily includes *CYP1A1* and *CYP1A2*, both of which are inducible upon exposure of the host to polycyclic aromatic hydrocarbons, arylamines, and dioxins [Dipple, 1983; Sugimura and Shigeaki, 1983; Nebert and Gonzalez, 1987; Whitlock, 1987].

The gastrointestinal tract is the primary tissue exposed to ingested pre-carcinogens (e.g.,

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polycyclic aromatic hydrocarbons) [Kaminsky and Fasco, 1992] and in particular, the small intestine and colon are able to metabolize and activate ingested pre-carcinogens [Stohs et al., 1977; Autrup et al., 1978; Fang and Strobel, 1978]. Induction of *CYP1A1* has been demonstrated for the rat small intestine and colon [McDanell and McLean, 1984; Traber et al., 1992]. Induced expression of *CYP1A2* was also detected in the rat small intestine [Traber et al., 1992]. Cytotoxicity and metabolism of the polycyclic hydrocarbons, benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene in established rat small intestinal epithelial cells (IEC) have been demonstrated [Quaroni and Isselbacher, 1981]. Induction of *CYP1A1* expression by polycyclic aromatic hydrocarbons and dioxin has also been shown in human colon, adenocarcinoma cell lines, CaCo-2 and LS174T [White et al., 1991; Boulenc et al., 1992].

Although the small intestine is capable of activating ingested pre-carcinogens, the incidence of cancer in this tissue is rare [Perzin et al., 1984]. Experimentally induced cancer of the small bowel is also infrequent [Gennaro et al., 1973]. However, activated pre-carcinogens formed in the small intestine may ultimately target susceptible tissues such as the colon. Colon cancer occurs with high frequency in the United States [Weisburger et al., 1977]. Pre-carcinogens in the diet that become activated in the small intestine and colon may be a significant factor in the development of colon cancer [Berg et al., 1973; Weisburger et al., 1977]. The presence of activated pre-carcinogens in the gut may also contribute to the greater occurrence of colon cancer seen in individuals with certain heritable diseases of the colon (e.g., familial polyposis) [Berg et al., 1973; Weisburger et al., 1977].

We have investigated the induction of *CYP1A1* expression in cultured rat small intestinal epithelial cells (IEC) [Quaroni et al., 1979; Quaroni and May, 1980], rat colonocytes (C3) [Celano et al., 1993], and human colon adenocarcinoma cells (CaCo-2) [Fogh et al., 1977] grown on tissue culture plastic or Matrigel, artificial basement membrane. IEC have been shown to have the properties of normal, proliferating, non-terminally differentiated intestinal crypt cells [Quaroni et al., 1979; Quaroni and May, 1980]. CaCo-2 cells, after 20–35 days in culture, differentiate to columnar, polarized epithelial cells with many of the characteristics of mature small intestinal enterocytes, e.g., expression of sucrase-isomaltase and microvilli [Pinto et al., 1983], C3 cells are a non-transforming, immortalized rat colonocyte cell line [Celano et al., 1993].

IEC and CaCo-2 cells grown on Matrigel did not proliferate, developed an altered morphology, became apoptotic and expressed the differentiation-specific enzyme sucrase-isomaltase. C3 cells cultured on Matrigel also had altered morphology, significantly reduced proliferation and increased apoptosis. Our results indicated that in differentiated intestinal epithelial cells, *CYP1A1* was selectively induced by benzo[*a*]pyrene and dioxin. We propose that the IEC, C3, CaCo-2, and other intestinal cell lines cultured on Matrigel provide cell culture models for the study of the role of *CYP1A1*-mediated activation of pre-carcinogens, its regulation in non-differentiated vs. differentiated cells and the molecular mechanism(s) by which activated pre-carcinogens mediate colon cancer.

MATERIALS AND METHODS

The following materials were obtained from the indicated sources: pTKGH [Selden et al., 1986] from the Nichols Institute (San Juan Capistrano, CA) as a component of their human growth hormone, transient gene expression assay system. Minimal essential medium, Earle's balanced salt solution, Geneticin (G418), gentamycin, penicillin/streptomycin, trypsin, and phosphate-buffered saline, Opti-MEM® and Lipofectin® reagents were from Gibco BRL (Grand Island, NY). Fetal calf serum was from ICN Biomedicals (Costa Mesa, CA). Matrigel, basement membrane gel, was from Collaborative Biomedical Products (Bedford, MA). FITC-conjugated goat, anti-mouse IgG from Hyclone (Logan, UT), Fluormount-G from Fisher Biotech. Lab Tek, glass slide chambers from VWR Scientific. Bicinchoninic acid (BCA) protein assay reagent kit, from Pierce (Rockford, IL), B[*a*]P and TCDD, from Aldrich and Company (Milwaukee, WI) and Chemsyn Laboratories (Lenexa, KS), respectively; pyruvic acid, luciferin, 7-ethoxyresorufin, resorufin, dicumarol, β-glucuronidase and arylsulfatase from Sigma (St. Louis, MO), Tween-20 from Biorad (Richmond, CA), insulin, from Eli Lilly (Indianapolis, IN).

Plasmids

The thymidine kinase promoter construct, pTKGH and the rat *CYP1A1* promoter-luciferase construct, pMC0LUC$^-$ have been described previously [Selden et al., 1986; Sterling et al., 1993].

Cell Culture

The rat small intestinal epithelial cells, IEC-6 and IEC-17 [Quaroni et al., 1979; Quaroni and May, 1980] were obtained from American Type Culture Collection, Rockville, MD and as a gift from Dr. Andrea Quaroni, Cornell, University. IEC were maintained in Dulbecco's modified Eagle's medium with 4.5 g/L glucose, bovine insulin, 0.1 U/ml, and 5% charcoal-stripped, fetal bovine serum. The human colon adenocarcinoma cells, CaCo-2, [Fogh et al., 1977] were obtained from American Type Culture Collection, Rockville, MD, and maintained in minimal essential medium (Eagle) with non-essential amino acids and Earle's balanced salt solution, and 20% charcoal-stripped, fetal bovine serum. Rat colonocytes (C3) [Celano et al., 1993] were a gift from Dr. Paul Celano, Johns Hopkins University and were maintained in minimal essential medium, 0.5 mg/ml Geneticin, 110 mg/L pyruvic acid, penicillin/streptomycin, and 15% charcoal-stripped serum. Cells were cultured on either Matrigel, tissue culture plastic or glass slide chambers with or without Matrigel.

Transfection and Reporter Gene Assays

Transfection was carried out by Lipofectin Reagent-mediated DNA transfer as described by the manufacturer. One million cells were seeded onto 60-mm dishes with or without Matrigel coating. pTKGH (2 μg) was co-transfected with pMC0LUC (5 μg) to account for any differences in plate to plate transfection efficiency. Twenty hours after the addition of the Lipofectin–DNA mixture, fresh medium was added containing vehicle alone (toluene) as a control or TCDD (0.1 μM). Forty-eight hours later, the reporter gene activity was assayed in the transfected cells. Expression of the pTKGH reporter gene construct was assayed as the amount of growth hormone secreted in the cell culture medium as per the manufacturer's instructions (Nichols Institute). The luciferase assay was performed as previously described [Brasier et al., 1989].

Protein Determination

The protein concentration of the cell lysates was determined using the Pierce BCA Protein Assay Reagent and the protocol supplied by the manufacturer.

Apoptosis

DNA fragmentation in apoptotic cells was determined by ApopTag^{TM}, In Situ Apoptosis Detection Kits (Peroxidase and Fluorescein) Oncor (Gaithersburg, MD). Cell nuclei were also stained with bisbenzamide (Hoechst).

EROD Assay

EROD enzyme activity was determined directly in intact cells cultured on 96-well plates as described [Donato et al., 1993]. 2×10^4 cells were plated per well, with or without Matrigel. Twenty-four hours later, the medium was replaced with medium containing either vehicle (acetone or toluene) or 1.0 μM, B[*a*]P or 0.1 μM, TCDD. After an additional 24 h, the cells were washed with phosphate buffered saline (PBS) followed by the addition of 100 μl of culture medium containing 8 μM 7-ethoxyresorufin and 10 μM dicumarol. The cells were incubated for 30 min at 37°C at which time 75 μl aliquots were transferred to a 96-well plate. Sodium acetate buffer (25 μl of 0.1 M), pH 4.5, containing 15 Fishman units of β-glucuronidase, and 120 Roy units of arylsulfatase were added to each well. The samples were incubated at 37°C for a further 2 h and 200 μl ethanol were added to each of the samples followed by centrifugation at 3,000*g* for 10 min. A Cytofluor 2350 (Millipore) with 530-nm excitation and 590-nm emission filters was used to measure supernatant fluoresence. Similarly processed known amounts of resorufin (0–80 pmol/well) were used to generate a standard curve.

Immunofluorescence

Indirect immunofluorescence was carried out essentially as described [Landay et al., 1983]. IEC or CaCo-2 cells grown on glass slide chambers with or without Matrigel were washed with PBS and fixed with a 3.75% solution of paraformaldehyde for 15–30 min followed by treatment with 0.5% solution of Tween-20 in PBS. The fixed cells were washed with PBS and blocked with 5% bovine serum albumin in PBS. The fixed, blocked cells were incubated on ice with mouse, anti-rat, or anti-human sucrase-

isomaltase [Hauri et al., 1980] in PBS for 30 min. The cells were then washed and blocked prior to incubation on ice with fluorescein labeled second antibody for 30 min. Cells were then washed and mounted in Fluormount-G. Fluorescent staining was detected and recorded with a Zeiss photomicroscope III with epifluorescence.

Immunoblot Analysis

Total cell lysate (50 μg of total protein for control and B[*a*]P-exposed cells) was combined with an equal volume of 2 × SDS–Gel loading buffer (5% SDS, 125 mM Tris-HCl, pH 6.8, 25% glycerol, 0.0025% bromophenol blue, plus 2-mercaptoethanol) and separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [Laemmli, 1970]. The SDS–PAGE-separated proteins were transferred to PVDF membrane for 20 min at 75 mA in 10 mM CAPS. The membrane with transferred protein was blocked with 5% dry milk, Tris-buffered saline (TBS), followed by incubation with anti-P450c (recognizes both CYP1A1 and CYP1A2), CYP1A1 or CYP1A2 antibodies diluted in 5% milk, TBS, all gifts from Dr. Paul Thomas, Rutgers University. After further washing, the membrane was incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL) for 1 h, washed and the protein signal developed, using the Pierce SuperSignal West Pico Chemiluminescent Substrate kit (Pierce). The blot was stripped of primary and secondary antibodies with IgG elution buffer (Pierce). Stripped membranes were reprobed with rabbit polyclonal antibody to actin (Sigma) to account for gel loading errors or possible differences in the amount of protein transferred to the PVDF membrane.

RESULTS

IEC Cultured on Matrigel Differentiated to Villus-Like Mature Enterocytes Within 24–48 h

IEC cultured on glass slides formed a monolayer of cuboidal cells and did not express the differentiation-specific enzyme, sucrase-isomaltase (Fig. 1, Panel A). IEC grown on glass slides did not become apoptotic as indicated by lack of fluorescein labeling or peroxidase activity (Fig. 1, Panels C and E). IEC cultured on Matrigel migrated to form a trabecula, connecting tube-like structures containing apoptotic cells radiating from centers of non-apoptotic cells all of which expressed sucrase-isomaltase (Fig. 1, Panels B, D, and F). DNA from IEC cells grown on plastic did not show intranucleosomal degradation, indicative of apoptosis as determined by agarose gel electrophoresis,

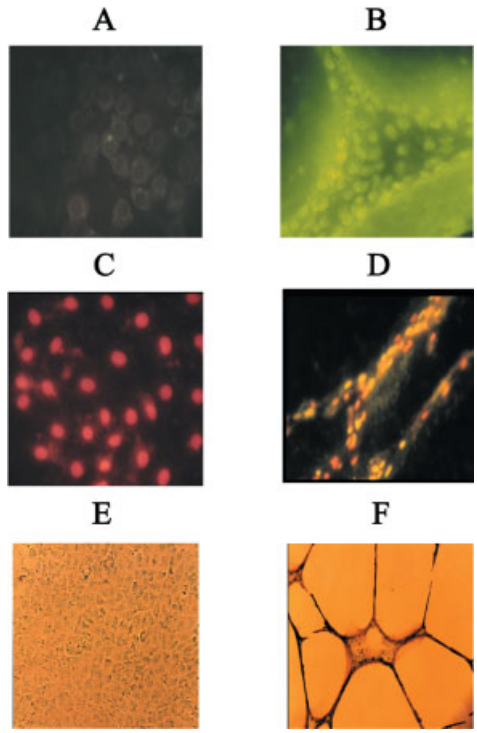

Fig. 1. Matrigel™-induced differentiation, morphology and apoptosis of rat intestinal epithelial cells. **Panel A**: Expression of the small intestinal epithelial cell differentiation-specific enzyme, sucrase-isomaltase was detected by Indirect-immunofluorescence. Rat IEC cultured on glass microscope slides for 48 h were reacted with mouse-anti-rat, sucrase-isomaltase, monoclonal antibody followed by FITC-linked goat anti-mouse IgG as the second antibody. Fluorescence (sucrase-isomaltase expression) was extremely weak or non-existent. **Panel B**: Same as Panel A except IEC cells were cultured on a Matrigel-coated glass microscope slide. Sucrase-isomaltase expression (fluorescence) was significantly induced and correlated with morphological change of the cells cultured on Matrigel. **Panel C**: Apoptosis was detected by ApopTag™ Plus In Situ, Apoptosis Detection Kit (Fluorescein). IEC were cultured on a glass microscope slide for 48 h, reacted with terminal deoxynucleotidyl transferase, digoxigenin-labeled nucleotide, anti-digoxigenin-fluorescein, and counter-stained with propidium iodide. Apoptosis was not detected in cell nuclei cultured on the glass microscope slide by this method. **Panel D**: Same as Panel C except IEC were cultured on a Matrigel-coated glass microscope slide. Apoptosis was significantly induced and correlated with morphological change of the IEC nuclei cultured on Matrigel. **Panel E**: Same as panel C except cells were reacted with anti-digoxigenin-peroxidase, diaminobenzidine and counter-stained with methyl green. **Panel F**: Same as Panel E except IEC was cultured on a Matrigel-coated glass microscope slide. Methyl green counter-stained cells are non-apoptotic and diaminobenzidine stained (dark brown) cells are apoptotic.

while IEC cells grown on Matrigel did (data not shown). The morphology of the IEC cells plated on Matrigel was similar to that previously reported for IEC-6 cells [Olson et al., 1991].

C3 Rat Colonocytes Changed Morphology Similar to IEC Cultured on Matrigel

C3 cells proliferated and had an elongated morphology when cultured on plastic or glass slides (Fig. 2, Panel A). Some apoptotic C3 cells could be seen piled on top of non-apoptotic C3 cells attached to plastic or glass (Fig. 2, Panel A). C3 cells cultured on Matrigel became oriented into tube-like structures containing apoptotic cells within 24–48 h similar to that of IEC cells on Matrigel (Fig. 2, Panel B). DNA from C3 cells grown on plastic did not show intranucleosomal degradation indicative of apoptosis while C3 cells grown on Matrigel did (data not shown).

CaCo-2 Cells Expressed a Significantly Greater Amount of Sucrase-Isomaltase Within 24–48 h When Cultured on Matrigel

CaCo-2 cells formed a monolayer when cultured on plastic or glass and expressed little or no sucrase-isomaltase within 24–48 h of plating (Fig. 3, Panel A). When cultured on Matrigel the CaCo-2 cells migrated to form isolated, densely packed clumps with occasional, connecting tube-like structures within 24–48 h and expressed a high level of sucrase-isomaltase (Fig. 3, Panel B). CaCo-2 cell nuclei were spheroidal when cultured on plastic or glass but became condensed and irregular when cultured on Matrigel as seen by staining with bis-benzamide (Fig. 3, Panels C and D, respectively). CaCo-2 cells cultured on plastic or glass were non-apoptotic 24–48 h after plating as indicated by a lack of peroxidase activity, while CaCo-2 cells cultured on Matrigel, become apoptotic within the same period of time (Fig. 3, Panels E and F, respectively). DNA from CaCo-2 cells grown on plastic did not show intranucleosomal degradation indicative of apoptosis, while CaCo-2 cells grown on Matrigel did (data not shown).

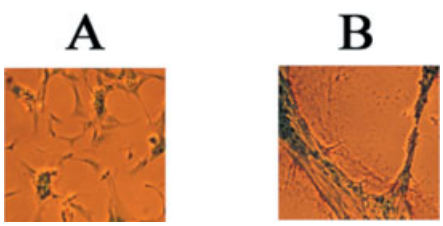

Fig. 2. Matrigel-induced apoptosis and morphology of rat colonocytes. **Panel A**: Rat colonocytes cultured on a glass microscope slide for 48 h, reacted with terminal deoxynucleotidyl transferase, digoxigenin-labeled nucleotide, anti-digoxigenin-peroxidase, diaminobenzidine, and counter-stained with methyl green. **Panel B**: Same as Panel A except rat colonocytes were cultured on a Matrigel-coated glass microscope slide.

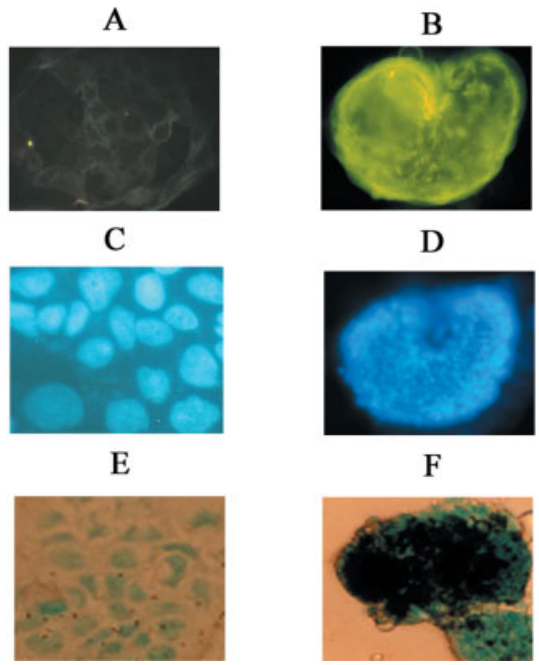

Fig. 3. Matrigel-induced differentiation, apoptosis and morphology of CaCo-2 cells. **Panel A**: Indirect-immunofluorescence of CaCo-2 cells cultured on a glass microscope slide for 48 h using as the first antibody, mouse-anti-human, sucrase-isomaltase, monoclonal antibody followed by FITC-linked goat anti-mouse IgG as the second antibody. **Panel B**: Same as Panel A except CaCo-2 cells were cultured on a Matrigel-coated glass microscope slide. **Panel C**: CaCo-2 cells were cultured on a glass microscope slide for 48 h and reacted with bis-benzamide (Hoescht). **Panel D**: Same as Panel C except CaCo-2 cells were cultured on a Matrigel-coated glass microscope slide. **Panel E**: CaCo-2 cells were cultured on a glass microscope slide for 48 h, reacted with terminal deoxynucleotidyl transferase, digoxigenin-labeled nucleotide, anti-digoxigenin-peroxidase, diaminobenzidine, and counter-stained with methyl green. **Panel F**: Same as Panel E except CaCo-2 cells were cultured on a Matrigel-coated glass microscope slide.

EROD Activity Was Inducible in Rat Small Intestinal Epithelial Cells and Colonocytes Cultured on Matrigel

There was no detectable induction of EROD activity by B[a]P or TCDD in IEC-6 or C3 cultured on plastic (Fig. 4, Top and Middle Panels). A 2- and 5-fold increase in EROD activity was observed in IEC cultured on Matrigel when exposed to B[a]P and TCDD, respectively (Fig. 4, Top Panel). C3 cultured on Matrigel had

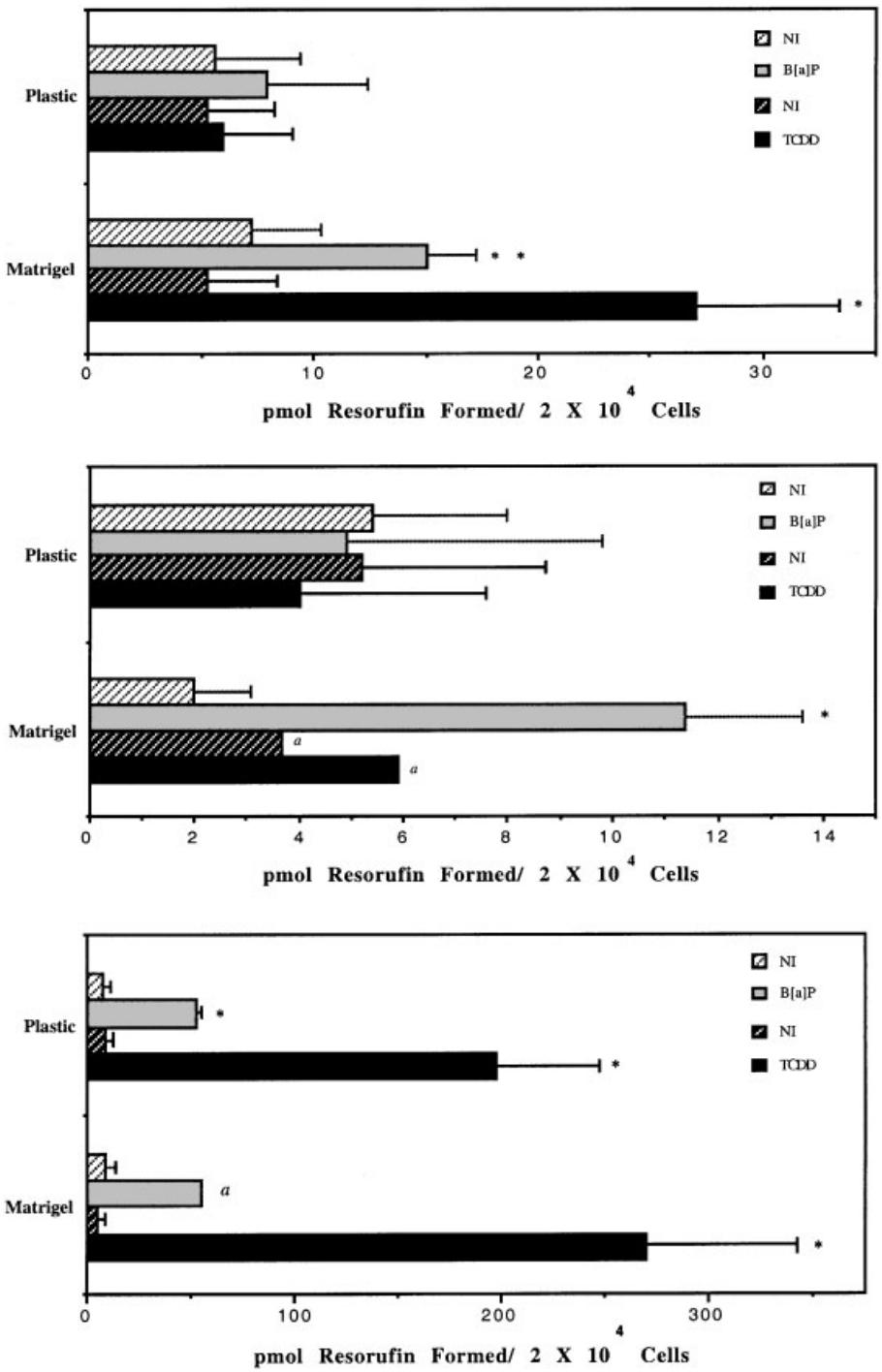

Fig. 4. **Top panel**: EROD activity of IEC seeded at a density of 2×10^4 cells onto a 96-well microtiter tissue culture plate with or without a Matrigel coating. Non-induced, NI (acetone or toluene vehicle only), 1.0 μM, benzo[*a*]pyrene-treated (B[*a*]P), 0.1 μM, 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin-treated (TCDD). Cells were exposed to B[*a*]P or TCDD for 48 h. Values are the mean ± SE of three experimental determinations. (**) indicates $P \geq 0.10$, (*) indicates $P \geq 0.05$ as determined by Student's non-paired *t*-test. **Middle panel:** EROD activity of C3 cells seeded at a density of 2×10^4 cells onto a 96-well microtiter tissue culture plate with or with out a Matrigel coating. Non-induced, NI (acetone or toluene vehicle only), 1.0 μM, benzo[*a*]pyrene-treated (B[*a*]P), 0.1 μM, 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin-treated (TCDD). Cells were exposed to B[*a*]P or TCDD for 48 h. Values are the mean ± SE of three experimental determinations. (*) indicates $P \geq 0.05$ as determined by Student's non-paired *t*-test. (a) indicates the mean of two experimental values (i.e., NI, 5.2, 2.2, and TCDD, 5.9, 5.9). **Bottom panel:** EROD activity of human adenocarcinoma cells (CaCo-2) seeded at a density of 2×10^4 cells onto a 96-well microtiter tissue culture plate with or with out a Matrigel coating. Non-induced, NI (acetone or toluene vehicle only), 1.0 μM, benzo[*a*]pyrene-treated (B[*a*]P), 0.1 μM, 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin-treated (TCDD). Cells were exposed to B[*a*]P or TCDD for 48 h. Values are the mean ± SE of three experimental determinations. (*) indicates $P \geq 0.05$ as determined by Students non-paired *t*-test. (a) indicates the mean of two experimental values (i.e., 64, 45).

induced EROD activities of 6- and 1.6-fold when exposed to B[*a*]P and TCDD, respectively (Fig. 4, Middle Panel). In the CaCo-2, human colon adenocarcinoma cells cultured on either plastic or Matrigel, EROD activity was induced 7-fold by B[*a*]P (Fig. 4, Bottom Panel). TCDD Induction of EROD activity in CaCo-2 cells cultured on Matrigel was 2-fold greater (i.e., 57-fold vs. 24-fold) than that for cells cultured on plastic (Fig. 4, Bottom Panel).

Rat *CYP1A1* Promoter-Luciferase Activity Was Induced in Transiently-Transfected IEC and C3 Cells Cultured on Matrigel

TCDD induced promoter activity of pMC0LUC– by 14- and 3-fold, respectively, in IEC and C3 cultured on Matrigel (Fig. 5). Induction of pMC0LUCby TCDD was not observed in IEC or C3 cultured on plastic.

CYP1A1 Protein Expression Was Induced by B[*a*]P in IEC Cultured on Matrigel But Not on IEC Cultured on Plastic

Immunoblot analysis using rabbit anti-CYP1A1 as the primary antibody indicated a detectable level of constitutive expression and B[*a*]P-inducible expression of CYP1A1 in IEC cultured on Matrigel (Fig. 6, lanes MC and MB, respectively). Constitutive or induced CYP1A1 expression was not detected in IEC cultured on plastic (Fig. 6, lanes PC and PB, respectively).

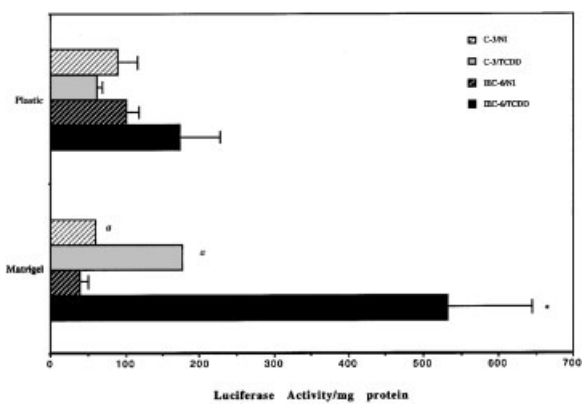

Fig. 5. Promoter (luciferase) activity of the rat *CYP1A1*-luciferase construct in transfected IEC-6 and C3 cells. 1×10^6 IEC or C3 cells were seeded onto 60 mm tissue culture dishes with or without Matrigel. After 24 h, the cells were transiently transfected using Lipofectin®. The Lipofectin–DNA-containing medium was replaced after 24 h with medium containing either vehicle (toluene) or inducer. The cells were assayed for luciferase activity 48 h later. Non-induced, NI (vehicle only), Induced, TCDD, (0.1 μM, 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin). Values are the mean ± SE of three experimental determinations. (*) indicates $P \geq 0.05$ as determined by Student's non-paired *t*-test. (a) indicates the mean of two experimental values (i.e., NI: 55, 64 and TCDD: 213, 138).

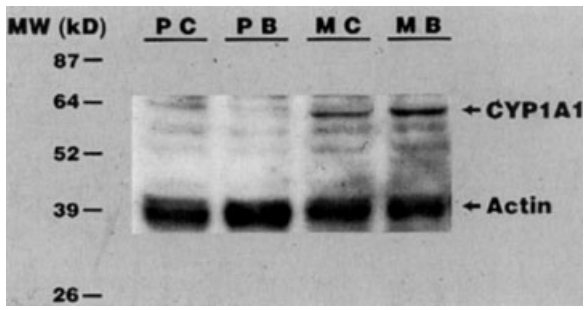

Fig. 6. Induction of CYP1A1 in Matrigel-differentiated rat intestinal epithelial cells. Immunoblot analysis of rat IEC cells (50 μg total cell protein each) for CYP1A1 expression was carried out with a rabbit polyclonal antibody specific for CYP1A1. PC: rat IEC cells cultured on plastic with vehicle alone for 24 h. PB: rat IEC cells cultured on plastic and treated with 4 μM benzo[*a*]pyrene (B[*a*]P) for 24 h. MC: rat IEC cells cultured on Matrigel with vehicle alone for 24 h. MB: rat IEC cells cultured on Matrigel and treated with 4 μM benzo[*a*]pyrene for 24 h. Actin expression was determined as a control for loading and transfer of cell protein.

CYP1A1 Protein (Approximately 52 kDa) Expression in CaCo-2 Cells Cultured on Plastic Was Not Induced by B[*a*]P, However, A Lower Molecular Weight (Approximately 39 kDa) Cross-Reacting Protein Was Induced by B[*a*]P

CaCo-2 cells cultured on plastic were grown to confluence (approximately 3–5 days) and treated with vehicle or 4 μM B[*a*]P for 48 h. Constitutive expression of CYP1A1 was observed in the untreated cells by immunoblot analysis, using a rabbit polyclonal antibody that recognizes both CYP1A1 and CYP1A2 (Fig. 7, Control lane). The approximately 52 kDa band observed in the immunoblot of CaCo-2 cells is CYP1A1 based on the Boulenc et al. [1992] report that there was no CYP1A2 enzyme activity, mRNA or protein detectable in CaCo-2 cells. A cross-reacting protein of approximately 39 kDa was observed in the untreated CaCo-2 cells that appears not to be a degradation product of CYP1A1 (Fig. 7, Control lane). CYP1A1 protein was not induced by exposure to B[*a*]P, but was decreased (Fig. 7, benzo[*a*]pyrene lane. Interestingly, the 39 kDa cross-reacting protein was induced by exposure to B[*a*]P (Fig. 7, Benzo[*a*]-pyrene lane). Boulenc et al. [1992] reported a lower molecular weight, cross-reacting protein to CYP1A antibody in CaCo-2 cells that was not induced by dexamethasone, phenobarbital or β-naphthoflavone which may be identical to

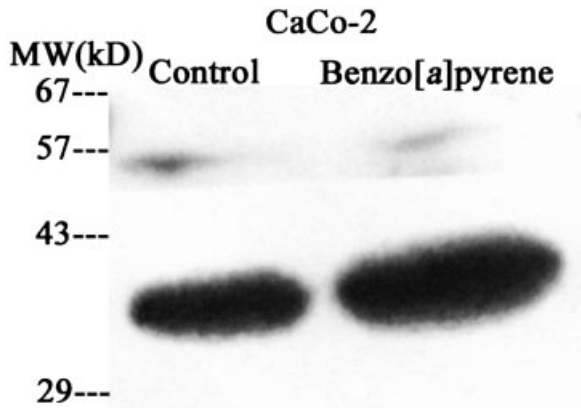

Fig. 7. Induction of CYP1A1 in human colon adenocarcinoma cells (CaCo-2) cultured on plastic. Immunnoblot analysis of non-treated (vehicle alone) and B[*a*]P (4 μM)-treated CaCo-2 cells cultured on plastic (50 μg total cell protein each) for CYP1A1 expression was carried out with a rabbit polyclonal antibody specific for CYP1A. There is no induction of the mature (approximately 52 kDa) species of CYP1A, however, a significant increase of a lower molecular weight (approximately 39 kDa) protein was detected in the B[*a*]P-treated cells.

the 39 kD cross-reacting protein observed in CaCo-2 cells shown in this report.

DISCUSSION

Colon Cancer and CYP1A

Colon cancer has a very high incidence in the United States [Weisburger et al., 1977]. Most colon cancers are sporadic while a small percentage are inherited, [Powell et al., 1993; Miyaki et al., 1995]. The high incidence of sporadic colon cancer in the United States can be attributed to dietary pre-carcinogens [Berg et al., 1973]. Activation of dietary pre-carcinogens by CYP1A enzymes in the liver may contribute to the occurrence of colon cancer [Weisberger, 1971], however, primary activation of ingested pre-carcinogens in the gut is of greater importance in the development of colon cancer, [Wattenberg, 1971; Kaminsky and Fasco, 1992]. Activated carcinogens from the diet can target any number of genes involved in tumor suppression and/or cell growth regulation in intestinal cells, e.g., APC and p53 tumor suppressor genes.

CYP1A and Intestinal Cells

An extensive study with CaCo-2 cells was carried out which demonstrated that *CYP1A1* and not *CYP1A2* was inducible in these cells [Boulenc et al., 1992]. CaCo-2 cells, although derived from a human colon adenocarcinoma, differentiate into small intestinal-like columnar epithelial cells (enterocytes) after 10–20 days or more in culture on plastic [Pinto et al., 1983, Boulenc et al., 1992]. Traber et al. [1992] showed that *CYP1A2* is inducible in the rat small intestine. Thus, it is possible that *CYP1A* regulation and induction in CaCo-2 cells may not be totally indicative of small intestinal enterocytes in vivo. However, the lack of induction of *CYP1A2* in these cells is in agreement with the lack of induction of *CYP1A2* in the rat colon [Traber et al., 1992]. Maximal expression of the differentiation specific enzyme, sucrase-isomaltase in CaCo-2 cells cultured on plastic occurred at approximately day 25 of culture [Pinto et al., 1983; Boulenc et al., 1992]. EROD (CYP1A1) activity was not detected until day 7 of culture on plastic and was maximally induced at days 15–25 of culture [Boulenc et al., 1992].

In the present study, culture of CaCo-2 cells on Matrigel resulted in significant expression of sucrase-isomaltase only 24–48 h after plating whereas, CaCo-2 cells cultured on plastic showed little or no expression of this differentiation specific enzyme within the same time period. A 2-fold greater induction of EROD (CYP1A) activity in CaCo-2 cells by dioxin was observed in this study after a total of 48 h in culture. Apparently, basement membrane components and/or other factors accumulate that trigger CaCo-2 cell differentiation into small intestinal-like enterocytes as the cells remain in culture on plastic. Culture on Matrigel accelerates the differentiation process seen at approximately day 20 of culture on plastic. Also, apoptosis of CaCo-2 cells is observed within 24–48 h when these cells are cultured on Matrigel.

EROD (CYP1A) activity was specifically induced by TCDD and to a greater extent than B[*a*]P in IEC-6 cultured on Matrigel, whereas in C3 cultured on Matrigel, B[*a*]P induced EROD activity to a greater extent. Promoter activity of the transfected rat *CYP1A1* construct, pMC0LUC-, was also induced to a significantly greater extent in IEC-6 cultured on Matrigel than in transfected C3 cells cultured on Matrigel. The difference in the levels of induction of *CYP1A1* appears to be cell type specific. CYP1A1 protein was constitutively expressed and was induced by exposure to B[*a*]P in IEC cultured on Matrigel, but not in IEC cultured on plastic as determined by immunoblot analysis. CaCo-2 cells cultured on plastic expressed CYP1A1 constitutively and its expression was

not induced by exposure to B[*a*]P. A lower molecular weight cross-reacting protein was induced by B[*a*]P exposure in CaCo-2 cells. The identity of this protein is unknown, but may be identical to that observed in CaCo-2 cells by [Boulenc et al., 1992].

Basement Membrane and Differentiation

Matrigel, basement membrane matrix consists of laminin, collagen IV, entactin, and heparin sulfate proteoglycan as well as growth factors and other components [Kleinman et al., 1986]. Matrigel mimics the specialized extracellular matrix layer found at the baso-lateral surface of intestinal epithelial cells [Simon-Assmann et al., 1986]. Hepatocytes cultured on Matrigel maintain differentiated function [Bissell et al., 1987]. Also, mouse mammary epithelial cells differentiate and express casein when cultured on Matrigel [Li et al., 1987; Barcellos-Hoff et al., 1989]. We have demonstrated that IEC-17, IEC-6 (IEC), and CaCo-2 cells cultured on Matrigel differentiated to mature small intestinal-like enterocytes and became apoptotic within 48 h. In this present study, *CYP1A1* was specifically induced in differentiated IEC and CaCo-2 cells. The importance of this observation is that we have a model system to elucidate differentiation-specific factors and/or non-differentiated cell factors that allow or suppress the induction of this important carcinogen metabolizing enzyme. Since, we did not examine C3 cells cultured on Matrigel for a differentiation-specific phenotype, it is assumed that due to their decreased rate of proliferation and altered morphology, they also were differentiated-like. Nevertheless, EROD activity (CYP1A) was specifically induced in C3 cells cultured on Matrigel. In addition, CaCo-2 cells cultured on Matrigel had a 2-fold greater induction of EROD activity mediated by dioxin compared to CaCo-2 cells grown on plastic.

Apparently, components of basement membrane, Matrigel, affect differentiation of IEC [Carroll et al., 1988; Olson et al., 1991; present study) and CaCo-2 cells [Lorentz et al., 1997; present study]. The migration of IEC-6 to form multicellular structures when cultured on artificial basement membrane was inhibited by antiserum to laminin or addition of the pentapeptide, Tyr-Ile-Gly-Ser-Arg an inhibitor of cell attachment to laminin [Olson et al., 1991]. More recent investigations of the role of laminin have further indicated that it is a key basement membrane component involved in CaCo-2 cell differentiation as well [Lorentz et al., 1997]. Antiserum to collagen IV did not inhibit IEC-6 from forming multicellular structures when cultured on artificial basement membrane [Olson et al., 1991], indicating that collagen IV may not participate in the Matrigel-induced altered morphology of IEC-6. Cell membrane integrins that function as receptors for laminin also play an important role in the differentiation process [Lorentz et al., 1997; Orian-Rousseau et al., 1998]. TGF-β, a cytokine present in Matrigel, may contribute to the differentiation of IEC-6 (e.g., sucrase-isomaltase expression), however, there are conflicting reports [Kurokowa et al., 1987; Barnard et al., 1989]. In our hands, exposure of IEC or CaCo-2 cells cultured on plastic or glass to TGF-β did not induce sucrase-isomaltase expression, morphological change, or apoptosis (data not shown).

Homeobox Gene Expression, Intestinal Differentiation and CYP1A

We examined homeobox gene expression in the intestine of suckling rats after administration of glucocorticoid. Glucocorticoid administration induced differentiation of the intestine as indicated by expression of sucrase-isomaltase, [Walsh et al., 1987a]. In addition, a homeobox-like mRNA was markedly increased in glucocoticoid-treated suckling rat intestine [Walsh et al., 1987b]. It was also demonstrated that the basement membrane components, collagen type IV and laminin were increased in the suckling rat intestine as a result of glucocorticoid administration [Walsh et al., 1987a]. More recent studies have shown that expression of the intestine-specific *caudal*-related homeobox gene product Cdx2, results in differentiation and induction of sucrase-isomaltase in rat IEC and CaCo-2 cells [Suh et al., 1994; Suh and Traber, 1996; Lorentz et al., 1997]. Cdx2 triggers expression of sucrase-isomaltase by binding to a specific element in its promoter [Suh et al., 1994; Suh and Traber, 1996], as well as the promoters of other enterocyte specific genes [Levy et al., 1995; Drummond et al., 1996; Lambert et al., 1996; Lee et al., 1996; Troelsen et al., 1997]. We suggest that Cdx2 is also capable of regulating *CYP1A* expression in IEC and CaCo-2 cells.

The present report indicates that IEC, C3 and CaCo-2 cells cultured on Matrigel, are model systems to study the regulation of *CYP1A1*

expression and dietary pre-carcinogen activation in the gut and its relationship to development of colon cancer. Basement membrane and signal transduction factors that mediate intestinal epithelial cell differentiation and apoptosis can be studied in this cell culture system.

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