Development of Caco-2 Cells Expressing High Levels of cDNA-Derived Cytochrome P4503A4

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Purpose. To develop Caco-2 cell derivatives expressing high levels of human cytochrome P450 drug metabolizing enzymes.
Methods. The cDNAs for two cytochrome P450 forms, CYP2A6 and CYP3A4, were introduced into an extrachromosomal vector under control of the cytomegalovirus early intermediate promoter. Vector-bearing cells were selected via resistance to hygromycin B.
Results. Transfected cells exhibited high levels of cDNA-derived protein as measured by Western blot, spectrophotometric P450 determination and/or cytochrome P450 form-selective enzyme assay. CYP3A4 and CYP2A6 catalytic activities were about 100 fold higher than in control cells. cDNA-expressing cells were found to form tight monolayers and were suitable for study of xenobiotic transport and metabolism. The permeabilities of cephalexin, phenylalanine, mannitol and propranolol across transfected monolayers were found to be similar to those across untransfected monolayers. The appropriate transfected monolayers metabolized the CYP2A6 substrate coumarin and the CYP3A4 substrates testosterone and nifedipine.
Conclusions. A Caco-2 cell system to simultaneously study drug transport and metabolism has been developed.

KEY WORDS: Caco-2; cytochrome P450; transfection.

INTRODUCTION

Identification of drug candidates that are permeable across the intestinal membrane and resistant to first-pass metabolism is an important step in oral drug development. Because our understanding of the relationships between structure and permeability as well as between structure and metabolic potential is not sufficient to predict the outcome, empirical screenings are routinely performed to select the best possible candidates for further development.

Caco-2 monolayers are routinely used as a permeability screening model (1–4). Caco-2 cells spontaneously differentiate and develop the properties of mature intestinal cells. This system has been shown to be useful for determining drug absorption via passive diffusion. A good correlation exists between permeabilities across Caco-2 cells and human bioavailabilities *in vivo* (4).

The Caco-2 system, as with any other *in vitro* model systems, is not a perfect model of intestinal epithelium. For example, Caco-2 cells do not produce mucus and appear to express insignificant levels of drug metabolizing enzymes of the cytochrome P450 class, which are found at high levels in the human intestine. Intestinal and hepatic cytochrome P450 (CYP)-associated drug-drug interactions can affect therapeutic outcome by inhibiting first pass metabolism and allowing higher than desired drug concentrations in plasma (e.g. cyclosporins (5)) or inhibiting metabolism of a prodrug (i.e. terfenadine (6)). CYP3A4 appears to be the principal cytochrome P450 enzyme in human intestine. It has been discovered that intestinal cytochrome P450s contribute significantly to the first pass metabolism of many drugs (7–9). It appears that some drug-drug interactions also occur at the level of intestinal metabolism. The CYP3A4 protein level in intestine has not been rigorously quantitated (due, in part, to the difficulty in preparing microsomes from intestinal tissue). Based on immunostaining, the CYP3A4 protein level in intestinal epithelium appears to be comparable to the level found in liver (9). It should be noted that catalytic activity will also depend on NADPH cytochrome P450 oxidoreductase (OR) activity and that both apoprotein and holoenzyme may be detected antigenically.

We have utilized an extrachromosomal vector, based on the OriP and EBNA-1-encoding sequences from Epstein Barr virus to express CYP2A6 and CYP3A4 cDNAs in Caco-2 cells. CYP2A6 was chosen because it has a simple enzyme assay which can be conducted in whole viable cells. This facilitated initial development and characterization of the culture conditions. CYP3A4 was chosen because this is the principal cytochrome P450 enzyme present in intestine.

MATERIALS AND METHODS

Stock Cell Culture

Caco-2 cells were propagated in DMEM supplemented to 10% with heat inactivated fetal bovine serum in plastic tissue culture flasks. Untransfected cells were routinely passaged by trypsinization with a 1:10 split once a week. All cultures were refed with fresh media every 2 to 3 days. Vector bearing cells were routinely passaged with a 1:6 split (vector without cDNA) or 1:2 to 1:4 split (CYP3A4-expressing cells). Selection for the vector was maintained by the addition of 100 μg/ml hygromycin B to the culture media. Cell attachment after trypsinization was found to be facilitated by initial plating in medium without vector selection; hygromycin B selection was added two days after plating. For the cDNA-expressing cell lines in general and for the CYP3A4-expressing derivatives in particular, cell reattachment after trypsinization was low (estimated at <20% for CYP3A4-expressing cells even with delayed addition of hygromycin B).

DNA Manipulations

Plasmid expression vectors were constructed using standard protocols. We utilized an episomal system based on the OriP sequences for Epstein Barr virus and the EBNA-1 gene product (10). The backbone vector, p220.2 was obtained from Dr. Bill Sugden of the University of Wisconsin under license from the Wisconsin Alumni Research Foundation. The isolation of the CYP2A6 cDNA was described previously (11). The CYP3A4 cDNA was obtained from Dr. F. J. Gonzalez and Dr. H. V. Gelboin of the National Cancer Institute and we have previously expressed it in human lymphoblasts (12).

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Caco-2 cells were transfected by electroporation. After electroporation, cells were replated and allowed to recover for 2 days prior to the addition of hygromycin B selection. The initial hygromycin B concentration was 400 μg/ml and was maintained for one week. The hygromycin B concentration was then decreased to 100 μg/ml. Cells transfected with CYP2A6-bearing vector were subjected to hygromycin B selection as a bulk culture only. Cells transfected with CYP3A4-bearing vector were subjected to hygromycin B selection B as a bulk culture and in addition, putative clonal derivatives were isolated by colony formation at limiting dilution in 24 well plates.

Cell Fractionation and Enzyme Assays with Cell Fractions

Caco-2 cells were fractionated to prepare a microsomal fraction according to Penman *et al.* (13). Cytochrome P450 content was determined according to Omura and Sato (14) Cytochrome C reductase was measured according to Langdon and Phillips (15). Coumarin 7-hydroxylase activity (16, 17) was measured by adding 50 μM coumarin to the culture media and incubating at 37 C for several hours. A portion of the culture media was then added to 0.1 M Tris pH 9 and the fluorescence measured with excitation at 368 nm and emission at 456 nm using a Farand Spectrofluorometer. Testosterone metabolism was studied by a modification of the HPLC method of Waxman *et al.* (18). Protein concentrations for microsome preparations were performed according to Lowry *et al.* (19).

CYP3A4 apoprotein was detected by Western Immunoblotting. Primary antibody was developed to goats using rat CYP3A2 as immunogen (Daiichi Pure Chemicals Co., Ltd, Tokyo, Japan). Secondary antibody was alkaline phosphatase linked anti-goat lgG, developed in rabbits. The nitrocellulose was developed with nitroblue tetrazolium and 5-bromo-3-chloror-3-indolylphosphate developing solution at room temperature until bands appeared (5 to 15 minutes).

Transport Studies

Caco-2 cell monolayers were grown as described previously (2, 20). The quality of the monolayers was determined by measuring the transepithelial electrical resistance (TEER) (normally 400–800 ohm cm^2) and the permeability of [^{3}H]-mannitol (normally 0.69 × 10^{-6} cm/sec). The transcellular transport experiments were performed in the same way as described previously, using a diffusion chamber that holds a cell monolayer at the center of the chamber (20, 21). At the end of a cephalexin or phenylalanine experiment, the apical side was spiked with [^{3}H] labeled mannitol (final concentration 0.1 mM) and the transport of mannitol was monitored for 30 min. Cephalexin and phenylalanine were measured by previously described HPLC methods (20–22), whereas propranalol and mannitol were measured with liquid scintillation counting. Total cellular protein was assayed according to Bradford's method (23), after cell monolayers were disrupted with 0.1% Triton X-100 as described previously.

Metabolism studies were performed using cell monolayers grown on polycarbonate membranes. After substrate (1 mM coumarin, 0.2 mM testosterone, 0.2 mM nifedipine) was added to the AP side, experiments proceeded for 3 hr at 37°C and 50 rpm in a temperature controlled orbital shaker. Samples were taken from both the AP and BL sides at the end of the 3 hr incubation and the amount of metabolite was measured.

RESULTS

Cell Line Development and Characterization

We evaluated derivatives of the p220.2 episomal vector containing human CYP2A6 and CYP3A4 cDNAs under control of the Cytomegalovirus early intermediate (CMV) promoter. This class of vector, but lacking the EBNA-1 gene, has been used to successfully express a range of cytochrome P450 enzymes in human lymphoblasts (11–13, 24). Episomal vectors do not integrate into the host genome and are maintained as extrachromosomal DNA when the cells are propagated in the presence of appropriate selection for the vector (25).

Caco-2 cells were transfected with the CYP2A6-bearing vector designated p220CMV2A6. Bulk hygromycin B resistance cells were selected and expanded. Cells containing p220CMV2A6 exhibited elevated coumarin 7-hydroxylase activity, a CYP2A6-catalyzed biotransformation. Bulk resistant cells contained coumarin 7-hydroxylase activities of 8.7 pmole per (million cells × minute). This activity was not detectable in control cells (with or without the backbone, p220.2 vector). This level of activity is comparable to that reported for human lymphoblasts transfected with related vectors bearing the CYP2A6 cDNA (11).

Culture conditions are known to affect the level of expression from endogenous genes and transfected DNA. Therefore we determined whether addition of two known modulators of gene expression, sodium butyrate and phorbol 12-myristate 13-acetate (TPA) affected the levels of cellular coumarin 7-hydroxylase activity. The heme precursor aminolevulinic acid (ALA) was also added at 10 μg/ml to support heme synthesis. These determinations were conducted in recently confluent cells in 24 well plates. Sodium butyrate and TPA were examined individually and in combination. Both TPA and sodium butyrate increased expression of CYP2A6 from the CMV promoter and the effect of the combination treatment was approximately multiplicative for the CMV promoter. The maximum fold increases in activity were 6.5, 14 and 76 for butyrate, TPA and the combination of the two agents. These two agents were also examined for their effect on expression levels in cell monolayer, the results will be discussed below.

In order to further characterize the CYP2A6 enzyme produced by Caco-2 cells, microsomes were prepared from cells bearing p220.2 (control) and cells bearing p220CMV2A6 (91 days post transfection). Both sets of cultures (5, 175 cm^2 flasks) were pretreated with 4 mM sodium butyrate, 10 μg/ml ALA and 100 nM TPA. Coumarin 7-hydroxylase activity was measured in the microsomes the activities were 450 pmol/(mg min) and 4.6 pmol/(mg min) for cells bearing p220CMV2A6 and control cells respectively. The level of coumarin 7-hydroxylase activity in cells containing p220CMV is comparable to that reported for human liver microsomes (26). Cytochrome P450 spectra was not detectable in CYP2A6-containing microsomes (limit of detection approximately 20 pmol/mg).

We also incorporated human CYP3A4 cDNA into a p220.2 derivative with the CMV promoter controlling CYP3A4 expression. The resulting plasmid was designated p220CMV3A4. Caco-2 cells were transfected with this plasmid and both bulk

hygromycin B resistant cells and clonal isolates (based on colony formation in multiwell plates) were isolated and expanded to permit characterization. The bulk population appeared to be derived from approximately 200 independent colonies. In addition, several putative clonal isolates were characterized.

CYP3A4 expression levels were analyzed as testosterone 6β-hydroxylase activity, a CYP3A4-catalyzed reaction, in newly confluent cells (50–62 days post transfection) after 100 nM TPA and 4 mM sodium butyrate treatment. Activity levels ranged from 231 to 340 pmol/(mg cellular protein min) and were very similar between bulk selected cells and three clonal isolates. Very little clone to clone variability is typical of extrachromosomal vectors (27) and reflects the fact that expression level is determined by vector copy number and not integration site (since the vectors rarely integrate).

We found that cell reattachment after trypsinization was markedly reduced for CYP3A4-expressing cells. Clones 5 and 20 were found to grow very poorly and were not characterized further. Clone 4 and bulk transfected cells were further expanded for microsome preparation and quantitation of CYP3A4 catalytic activity and content. Microsomes were prepared from 10 to 12 175 cm^2 flasks of cells (101–104 days post transfection) which had been treated with 4 mM sodium butyrate, 10 μg/ml ALA and 100 nM TPA. CYP3A4 catalytic activity was again measured using testosterone 6β-hydroxylase activity, cytochrome P450 content was measured by reduced carbon monoxide difference spectra and CYP3A4 expression was also analyzed by Western Immunoblotting. Table I contains the results.

Caco-2 cells bearing p220CMV3A4 had a spectrophotometric cytochrome P450 content of 45 to 49 pmol/mg microsomal protein. The specific content was comparable to that achieved in human lymphoblasts expressing both CYP3A4 cDNA and cDNA-derived human cytochrome P450 oxidoreductase (OR). However, the level of CYP3A4-catalyzed testosterone 6β-hydroxylase activity was significantly lower in Caco-2 microsomes. The turnover of testosterone per unit enzyme was lower in Caco-2 cells relative to human lymphoblasts co-expressing CYP3A4 and OR. This observation implies that OR may be rate limiting for cytochrome P450 catalytic activity. The possibility of OR limiting CYP3A4 catalytic activity is supported by the levels of OR enzyme as measured by cytochrome C reductase activity. The levels in Caco-2 cell microsomes were 35 to 50 nmol/(mg min), substantially less than in human liver microsomes (28) or in the human lymphoblasts co-expressing CYP3A4 and OR (250 nmol/(mg min)). The level of OR in the intestinal epithelium is unknown.

Table I. Testosterone 6β-Hydroxylase and Cytochrome P450 Contents for Microsomal Fractions Prepared from Caco-2 cells Bearing the CYP3A4-expression Plasmid p220CMV3A4 and Human Lymphoblasts Co-Expressing CYP3A4 and OR

Cell Source	Testosterone 6β-Hydroxylase Activity (pmol/mg min)	P450 Content (pmol/mg)	Turnover number (per min)
Caco-2 Untransfected	8	Not detectable	NA
Caco-2 p220CMV3A4-Bulk	634	49	13
Caco-2 p220CMV3A4-clone 4	983	45	22
Lymphoblast-CYP3A4+Reductase	2200	60	37

Note: Microsome preparation, testosterone 6β-hydroxylase activity determinations and P450 content determinations were performed as described in the Materials and Methods section. Human lymphoblast microsomes were obtained from Gentest Corporation (Catalog No. M107r).

CYP3A4 protein levels in microsomes prepared from Caco-2 cells bearing p220CMV3A4 were also compared to CYP3A levels in human liver microsomes (HLM) by Western Immunoblots. Blots were probed with an anti-rat CYP3A2 antibody (which crossreacts with human CYP3A4) and developed with an alkaline phosphatase-linked secondary antibody. The blot is contained in Figure 1.

The relative Western blotting intensities for the Caco-2 cell microsomes was consistent with the relative levels of testosterone 6β-hydroxylase activity. Calculation of the substrate turnover numbers in Caco-2 microsomes indicates that the rate of substrate turnover is low per unit enzyme. Our experience with human lymphoblast expression of this (and other P450 enzymes) indicates that substrate turnover can be increased 3 fold by co-expression of P450 reductase (i.e. reductase levels are limiting enzyme activity).

As determined by Western blot, the CYP3A4 apoprotein levels we have obtained in Caco-2 cell microsomes here is roughly equivalent to that found in the human liver microsome samples. However, the testosterone 6β-hydroxylase activity in the transfected Caco-2 cell microsomes is approximately 20 to 50% of that found in human liver microsomes (2000–3000 pmole/(mg min)). This further indicates that the CYP3A4 holoenzyme in Caco-2 cells is somewhat less catalytically active than that in human liver. Activity limitation due to low OR levels in Caco-2 cells is the likely mechanism. The specific catalytic activity of the CYP3A4 (per unit enzyme) present in intestinal epithelium is also not known.

The levels of cDNA-derived CYP3A4 activity were found to decrease as a function of time of growth in cell culture. When weekly 1:4 passages were used, the activity decreased about 5 fold after five passages. Similar losses in activity were observed for bulk transfected cells and for clone 4. Concomitant with the reduction in CYP3A4 activity, cell growth improved. This implies a selective growth advantage to cells expressing

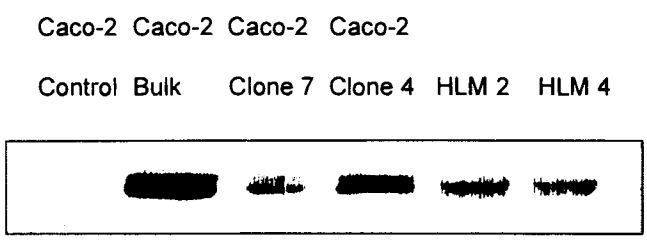

Fig. 1. Western Immunoblot of microsomes prepared from Caco-2 cells. Microsomal fractions were prepared from Caco-2 cells bearing p220CMV3A4 and control cells. Gel load was 25 μg protein per lane. For comparison purposes, a similar loading of human liver microsomes was analyzed side by side. Methods are described in the Materials and Methods section.

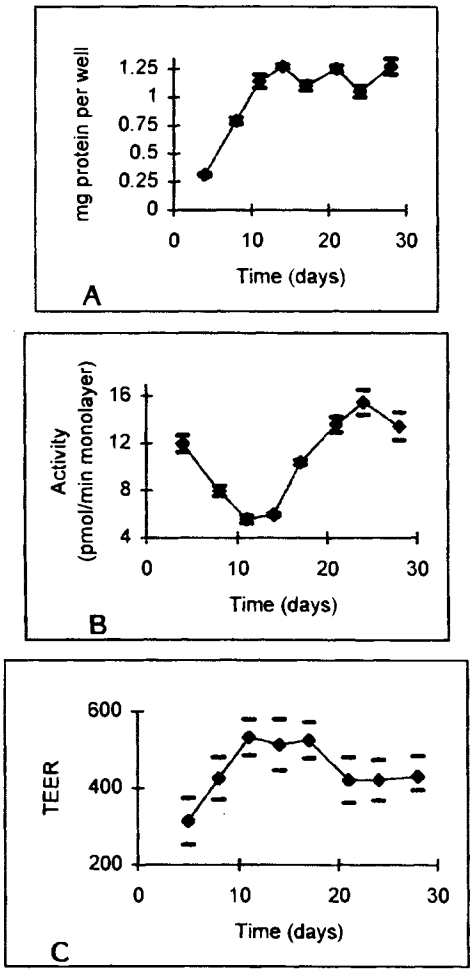

Fig. 2. Time dependence of total cellular protein (Panel A), CYP2A6-catalyzed coumarin 7-hydroxylase activity (Panel B) and TEER (Panel C) for Caco-2 cells bearing p220CMV2A6 when cultured in Milliwells™.

lower CYP3A4 levels. The mechanism for this loss of activity is unclear at this time. Such instabilities have not been observed for the same two cDNAs when expressed in human lymphoblastoid cells (11–13). Routine utilization of these cells is still possible by use of a large freezer stock and routine re-establishment of seed cultures for monolayer formation.

Drug/Xenobiotic Transport Studies

We have performed a series of characterizations of the transport metabolism properties of the monolayers of transfected cells in order to verify that the cells remain suitable for absorption/metabolism studies. In particular, we verified that the cells still produce good quality monolayers and that model substrates were transported with the expected efficiencies. These studies were conducted with normal Caco-2 cells, cells bearing the backbone vector (p220.2) and cells bearing vectors with the CYP2A6 (70–98 days post transfectio) or CYP3A4 (142–170 days post transfection) cDNAs under control of the CMV promoter.

Figures 2A, 2B and 2C contain the time course of cellular protein amount, CYP2A6 catalytic activity (coumarin 7-hydroxylase), and the TEER values for cells bearing p220.CMV2A6 grown in Millicells™. Protein and TEER values reached a plateau after about 10 days. Upon differentiation into a monolayer in the Millicell™ CYP2A6 catalytic activity increases. CYP2A6 catalytic activity did not plateau until about 20 days. The data summarized in Table II below indicate that CYP2A6 is catalytically active in monolayers and not detectably active in cells bearing the backbone vector. Expression of CYP2A6 remains responsive to TPA (50 nM) without affecting the TEER. Responsiveness to TPA was less pronounced in monolayers (grown for two weeks on Millicells), which had higher basal catalytic activity, relative to the data obtained in recently confluent cells (about 5 days after passaging in flasks). Pretreatment with sodium butyrate caused the monolayers to become leaky. Exposure of transfected and untransfected Caco-2 cells to butyrate resulted in altered morphology.

We also determined, as a function of time, the expression of cDNA-derived CYP3A4 catalytic activity, total cellular protein and TEER values for Caco-2 cells bearing p220CMV3A4 clone 4 (Figures 3A, B, C). The results with the CYP3A4 expressing cell line were similar to those observed for the CYP2A6 expressing cell line. As with CYP2A6, the levels of CYP3A4 catalytic activity could be increased by pretreatment with TPA.

In the absence of TPA treatment, the rate of formation of oxidized nifedipine (5.60 ± 0.08 pmole/min mg protein) was lower than the rate of formation of 6β-hydroxytestosterone (18.3 ± 2.2 pmole/min mg protein). Overnight treatment with TPA increased nifedipine oxidation 7 fold and testosterone 6β-hydroxylation 2 fold. These experiments were conducted on different batches of cells and TPA inducibility does decline with time (as basal activity increases).

We also determined the rate of testosterone 6β-hydroxylation and nifedipine oxidation in the presence or absence of 100 μg/ml hygromycin B, after the cells were seeded in Millicells™. The results indicated that there were no significant differences between these two treatments, although the specific activity was slightly higher (3–15%) with hygromycin B treated monolayers. On the other hand, the untreated monolayers had slightly higher TEER values and more protein.

Table II. Monolayer Properties and CYP2A6 Catalytic Activity in CYP2A6-Expressing Caco-2 Cells

Plasmid/TPA	Days in Culture	TEER	7-Hydroxycoumarin Formation (pmol/(mg total protein min)
p220.2	21	571	not detectable
p220.2 + TPA	22	407	not detectable
p220.CMV2A6	20	417	26.3 ± 1.7
p220.CMV2A6 + TPA	20	466	50.0 ± 2.9

Note: TEER and coumarin 7-hydroxylase determinations were performed as described in the Materials and Methods section. p220.2 was the backbone vector, without cytochrome P450 cDNA insert or promoter/polyadenylation sequences associated with the cytochrome P450 cDNA insert. TEER values were measured three times per Millicell. The mean of three Millicells was denoted as the average. Standard deviations were typically less than 15%. Typical variations can be seen in Figures 2 and 3.

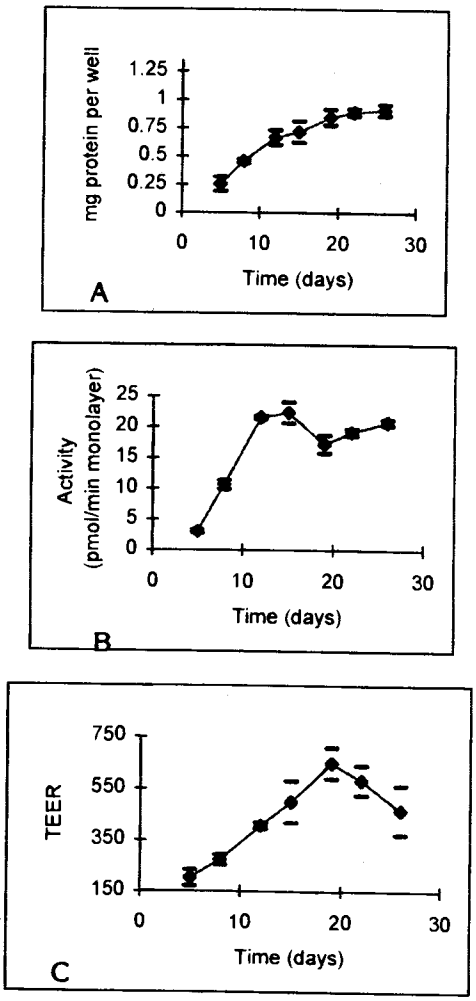

Fig. 3. Time dependence of total cellular protein (Panel A), CYP3A4-catalyzed testosterone 6β-hydroxylase activity (Panel B) and TEER (Panel C) for Caco-2 cells bearing p220CMV3A4 when cultured in Milliwells™.

The level of CYP3A4-catalyzed testosterone 6β-hydroxylation could be inhibited by preincubation of the cell monolayer with the prototypical CYP3A4 inhibitors trolenadomycin (TAO) and ketoconazole. A TAO concentration of 25 μM inhibited testosterone hydroxylation by 50%, 1 μM ketoconazole was completely inhibitory.

The permeabilities of 1 mM cephalexin and 1 mM phenylalanine, as well as mannitol and propranalol were also measured in cells bearing p220CMV2A6 and in cells bearing p220CMV3A4 (clone 4). The permeability experiments are summarized in Table III. cDNA-expressing and normal Caco-2 cells had comparable properties indicating that the presence of the cDNA/vector did not interfere with conducting transport studies with the cell line. The results indicated that, compared to untransfected cells, these cells have similar cephalexin permeability and TEER values. Cells bearing p220CMV3A4 had similar propranalol permeability, slightly higher mannitol permeability and significantly higher (72% more) phenylalanine permeability. Cells bearing p220CMV2A6 had lower propranalol and mannitol permeabilities and higher phenylalanine permeability.

DISCUSSION

We have successfully developed Caco-2 cells expressing cytochrome P450 cDNAs. The episomal vector based on the OriP sequences and EBNA-1 gene product derived from Epstein Bar virus provided relatively high expression levels of CYP2A6 and CYP3A4. Expression of CYP3A4 cDNA markedly affected cell growth. Cell reattachment frequency after trypsinization was reduced and cell growth rate also appeared reduced. Expression of cDNA-derived CYP3A4 was somewhat unstable, but this degree of instability could be addressed by the use of a freezer stock of freshly transfected cells. Clearly it would be desirable to have a more stable cell lines, however, at this time it is unclear whether instability of expression is a general properties of this cell line/vector system or limited to the cDNA(s) expressed in the present study. Additional development/characterization work is needed to clarify this point.

Comparison of Caco-2 cell expressed CYP3A4 catalytic activity per unit enzyme (activity relative to other systems) implies that OR activity may be limiting CYP3A4 catalysis. It may be possible to overcome this limitation by co-expressing OR cDNA with CYP3A4. In some mammalian systems high level OR expression is toxic to the host cells. Given the marked reduction in cell propagation observed with CYP3A4 expression, it is unclear whether efficient OR expression will be possible in this system.

Immunochemical evidence for CYP3A expression has been reported for some Caco-2 derivatives (29) and Caco-2 cells are reported to metabolize cyclosporin (30). Indeed, we have found a very low level of testosterone hydroxylation in untransfected Caco-2 cells. Microsomes from control cells produce a metabolite which co-chromatographs with 6β-hydroxytestosterone at an activity level of ~8 pmol/(mg microsomal protein min). This low level of activity effectively precludes analysis of metabolite formation for all but the most rapidly metabolized CYP3A substrates for which there are very sensitive analytical methods for metabolite detection. The preparation of microsomes achieves a 5 to 10 fold enrichment in cytochrome P450 enzymes (per unit protein) relative to whole cells. The testosterone hydroxylase activity in native Caco-2 cells is less than 1 pmol/(mg cellular protein min). The activity in CYP3A4-cDNA-expressing cells was about 100 fold higher than the native level. This higher level of expression should greatly simplify metabolite detection.

Even with the limitations with respect to stability and cell propagation discussed above, the CYP3A4-expressing cells have been found to be useful for drug transport and metabolism studies. The cells form tight monolayers (as witnessed by the TEER values and drug transport studies) and expressed native transporters. Because there are three major transport pathways (i.e. passive diffusion, paracellular leakage and carrier-mediated transport) for small xeno-/endo-biotics (MW < 1000 dalton), transport function of transfected Caco-2 cells was tested using propranalol, phenylalanine, mannitol and cephalexin. Propranalol was used to test the passive diffusion pathway, which is responsible for the transport of >90% of drugs. Mannitol was used to test the paracellular leakage pathway, as well as the integrity of the cell monolayers (because mannitol is not known to be taken up by these cells in any significant amount).

Table III. Monolayer Permeabilities of Normal and Transfected Caco-2 Cells

Parameter	Normal Caco-2 Cells	Cells with p220.CMV2A6	Cells with p220CMV3A4 (cl 4)
Days in Culture	20	17–18	13
TEER	473, 485, 568	512, 481, 546, 519, 480	551
Permeability d-mannitol (0.1 mM) (cm/sec × 10^6)	0.44 ± 0.03 0.45 ± 0.03	0.27 ± 0.06 0.22 ± 0.03	0.55 ± 0.15
Permeability cephalexin (1 mM) (cm/sec × 10^6)	3.0 ± 0.15	3.58 ± 0.30	3.0 ± 0.27
Permeability Phenylalanine (1 mM) (cm/sec × 10^6)	2.5 ± 0.3	3.2 ± 0.73	4.3 ± 0.45
Permeability propranalol (0.1 mM) (cm/sec × 10^6)	91.3 ± 3.4	62.9 ± 4.9	92.7 ± 1.7

Note: Permeability, TEER and catalytic activity determinations were performed as described in the Materials and Methods section. TEER values were measured three times per Millicell. The mean of three Millicells was denoted as the average. Standard deviations were typically less than 15%. Typical variations can be seen in Figures 2 and 3.

Quantitative properties of these two components (passive and paracellular) of absorption between wild-type and cDNA vector-bearing cells were compared to insure that the cells were similar and useful for the intended purpose. Phenylalanine and cephalexin were used to test the functions of carrier-mediated pathway via amino acid and peptide carriers. A concentration of 1 mM was used in order to permit reliable quantitation and to avoid saturation of the pathway (typical Km values are 2–5 mM). We chose the amino acid carrier since it transports drugs such as l-dopa, l-methyldopa and several other centrally active amino acid analog drugs. We chose the peptide carrier because this carrier is important for the absorption of peptide-like drugs such as oral β-lactam antibiotics and angiotensin-converting enzyme inhibitors. We also chose the peptide carriers because the expression of this transporter is sensitive to culture conditions. For example, it is differentially expressed by different strains of Caco-2 cells and may be affected by the presence of antibiotics in the media which significantly decrease the expression of the peptide carrier in Caco-2 cells (22). Therefore, this transporter represents one which is most likely to be adversely affected by transfection and potential presence of agents (in the media) for selection of the vector.

Studies with model compounds indicate that all three major transport pathways function normally in CYP2A6- or CYP3A4-expressing Caco-2 cells. CYP3A4 and CYP2A6 expressing cells were found to form monolayers which were sufficiency tight to permit conducting transport studies. The permeability results suggest that the characteristics of selected clones will differ somewhat from the original strain. This is not unusual in that cells from different labs also differ somewhat. The results with p220CMV3A4 also show that the monolayers obtained from the transfected cells are quite tight or we would have seen a more significant increase in phenylalanine permeability compared to the increase in mannitol permeability. The cells transfected with p220CMV2A6 were also quite tight as they had somewhat elevated phenylalanine permeability accompanied by a decrease in mannitol permeability.

The levels of catalytic activity in monolayers could be increased, without loss of membrane integrity, by pretreatment with TPA. Pretreatment with sodium butyrate caused the monolayers to become leaky. Exposure of transfected and untransfected Caco-2 cells to butyrate resulted in altered morphology. Thus the effect of butyrate on leakiness in transfected cells does not appear related to the accompanying increase in P450. Nevertheless, the levels of CYP3A4 appear to be sufficiently high and cell growth adequate to permit many application with the CYP3A4-expressing cells. Our studies of testosterone and nifedipine transport and metabolism support this conclusion.

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