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Expression of Multiple Cytochrome P450 Enzymes and Multidrug Resistance-Associated Transport Proteins in Human Skin Keratinocytes

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Cytochrome P450 enzymes metabolize various endogenous and exogenous small molecular weight compounds. Transport-associated proteins, such as P-glycoprotein, multidrug resistance-associated protein and lung resistance protein are overexpressed in drug-resistant cell lines, as well as in human tumors from various histologic origins, including malignant melanoma. Little is known about the expression and function of cytochrome enzymes and multidrug resistance-associated transport proteins in human skin; therefore, the aim of this study was to analyze the expression pattern of cytochrome enzymes and multidrug resistance-associated transport proteins in proliferating human epidermal keratinocytes under constitutive conditions and after induction with various inducers. Reverse transcription–polymerase chain reaction revealed constitutive expression of cytochromes 1A1, 1B1, 2B6, 2E1, and 3A5 in keratinocytes and showed expression of cytochrome 3A4 after incubation with dexamethasone. The expression of cytochrome 1A1 was enhanced on the mRNA level after induction with benzanthracene. Reverse transcription–polymerase chain reaction analysis of the multidrug resistance-associated transport proteins revealed constitutive expression of multidrug resistance-associated proteins 1 and 3–6, and lung resistance protein in human epithelial keratinocytes and was negative for multidrug resistance 1 and 2. Expression of 1 was seen after induction with dexamethasone. Reverse transcription–polymerase chain reaction results were confirmed by immunoblots which showed expression of cytochromes 1A1, 2B6, 2E1, and 3A, multidrug resistance-associated proteins 1, 3, and 5 as well as multidrug resistance 1 after induction with dexamethasone. Immunohistology showed positive immunofluorescence in skin specimens for cytochromes 1A1, 2B6, 2E1, and 3A and multidrug resistance-associated protein 1 and multidrug resistance 1. Constitutive activity of cytochrome 1A1, 2B, 2E1, and 3A enzymes was measured by catalytic assays. These results show that keratinocytes of the human skin express various transport-associated enzymes and detoxifying metabolic enzymes. Previous studies have revealed that cytochrome enzymes and transport-associated proteins play complementary parts in drug disposition by biotransformation (phase I) and anti-transport (phase III) and act synergistically as a drug bioavailability barrier. ***Key words: drug metabolism/drug transport/skin barrier. J Invest Dermatol 116:541–548, 2001***

Human skin is both a physical and a biochemical barrier to the absorption and penetration of potentially damaging environmental compounds. Beside the role of the stratum corneum as a most critical structure for epidermal barrier function there is increasing evidence indicating that xenobiotic metabolizing enzymes and transport proteins function as a second – biochemical – barrier of the skin (Jugert *et al*, 1994; Merk *et al*, 1996; Keeney *et al*, 1998b). The metabolic interaction between small molecular weight compounds and molecular targets in the cell can be separated at least in these phases: After penetration the xenobiotics are first chemically activated or inactivated by oxidative reactions (Guengerich, 1992). The most important family of enzymes involved in the reactions are the cytochrome P450 enzymes. In addition to their detoxifying functions they may be involved in allergic reactions to substances of low molecular weight, e.g., contact dermatitis. This has been shown for compounds such as eugenol, which is metabolized by 3-methylcholantrene inducible cytochrome P450 (Fischer *et al*, 1990). Eugenol is a sensitizer only in those mice which have inducible CYP 1A1 phenotype, giving further evidence that this enzyme system is important with regard to the formation of the related antigens and that polymorphism in the expression and inducibility of this enzyme may be important in individual risk of developing a sensitization (Merk *et al*, 1997, 1998;

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Abbreviations: CYP, cytochrome P450; MDR, multidrug resistance; P-gp, P-glycoprotein; MRP, multidrug resistance associated protein; LRP, lung resistance protein.

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Merk, 1998). In phase II the activated or inactivated metabolite is transformed by epoxide hydrolases, transferases and reductases such as NAD(P)H-quinone reductase, to convert the insoluble metabolite into a water-soluble substance, thus being able to be eliminated from the body, e.g., via urine (Lilienblum *et al*, 1986; Merk *et al*, 1991).

After selection for resistance to a single cytotoxic drug, cells may become cross-resistant to a whole range of drugs with different structures and targets, a phenomena called multidrug resistance (MDR; Kool *et al*, 1997). In human cancer cells, MDR can be caused by enhanced drug efflux mediated by transporter proteins such as the MDR1 P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), and lung resistance protein (LRP) (Borst *et al*, 1997). Expression of transport proteins, such as P-gp, has been found in normal epithelia, such as those of the gastrointestinal tract, liver, pancreas, kidney, and reproductive organs (Hunter *et al*, 1993). In epithelia and endothelia, P-gp is localized in the apical or luminal region of the cells. The polarized expression of P-gp has led to the suggestion that its physiologic role is as a secretory detoxifying system. Thus, in organs such as the kidney, pancreas, liver, and gastrointestinal tract P-gp would act to secrete toxins and for gastrointestinal epithelia also limit toxin absorption.

Many studies have investigated the interaction of CYP3A4 or orthologous CYP3A enzymes, and P-gp, which share many substrates and/or inhibitors (Kivisto *et al*, 1995). For example cortisol, dexamethasone, quinidine, and erythromycin, which are substrates of *in vivo* phenotyping assays for CYP3A4 are also substrates of P-gp (Ged *et al*, 1989; Wacher *et al*, 1995; Barnes *et al*, 1996; Kim *et al*, 1999). It has been postulated that CYP3A and P-gp in intestinal enterocytes may limit the bioavailability of drugs, with P-gp preventing diffusion of the parent drugs across the apical brush border membrane and CYP3A mediating their metabolism (Watkins, 1997). Synergism between both processes may occur when the metabolites produced by CYP3A are better substrates for P-gp than the parent drug (Kolars *et al*, 1992). Because CYP3A-mediated metabolism sometimes produces more toxic intermediates, P-gp-mediated efflux of reactive metabolites could also have important toxicologic consequences (Lewis *et al*, 1992).

As CYP3A and P-gp share several substrates and inhibitors, pharmacokinetic drug–drug interactions are often a composite, resulting from the interplay of both systems (Wacher *et al*, 1995; Benet *et al*, 1996; Lan *et al*, 2000). In one study, it was shown that the anti-fungal ketoconazole or cyclosporine A, which is used in dermatology for the treatment of psoriasis and atopic dermatitis, increased the area under the curve for vinca alkaloids most likely by inhibiting P-gp and CYP3A (Chan, 1998). Similarly, simultaneous inhibition of P-gp and CYP3A has been postulated to be responsible for the effects of ketoconazole on the bioavailability of digoxin or the cysteine protease inhibitor KO2 (Salphati and Benet, 1998; Zhang *et al*, 1998).

Skin is a major interface between the environment and the body; however, little is known about the expression and function of CYP enzymes and in particular of MDR-associated transport proteins in this tissue as well as role of their interaction in absorption and metabolism of xenobiotics. Previous studies revealed that P-gp has a physiologic function during the migration of dendritic cells from skin via lymphatic vessels (Randolph *et al*, 1998) and that keratinocytes show a high expression of LRP (Izquierdo *et al*, 1996) and CYP enzymes, such as CYP2B19 (Keeney *et al*, 1998a). Therefore, the aim of this study was to analyze the expression patterns of CYP enzymes and MDR-associated transport proteins in proliferating human epidermal keratinocytes under constitutive conditions and after induction with various inducers.

MATERIALS AND METHODS

Chemicals Keratinocyte basal medium, medium supplement kit, detach kit (Promo Cell, Heidelberg, Germany); 7-pentoxyresorufin, 7-ethoxyresorufin, resorufin (Pierce, Rockford, IL) bovine serum albumin, erythromycin, para-nitrophenol, 4-nitrocatechol (Sigma, St Louis, MO); NADPH, phosphate-buffered saline (PBS; Boehringer, Mannheim, Germany); nonfat milk powder, Tris (Bio-Rad, Richmond, VI); anti-CYP1A1, 2B, 2E1, and 3A rabbit (Oxygene, Dallas, TX), monoclonal anti-CYP2B6, 2E1 (Gentest, Woburn, MA); rabbit CYP1A1, 3A4 (Daiichi Pure Chemicals, Tokyo, Japan) (Fujino *et al*, 1982; Khan *et al*, 1987, 1988, 1989; Waxman *et al*, 1987; Gelboin *et al*, 1988, 1996); monoclonal anti-MDR1/C219 (Signet Laboratories, Dedham, MA) (Chan *et al*, 1988); monoclonal anti-MRP1/MRPm6 (Chemicon International, Temecula, CA) (Flens *et al*, 1994); monoclonal anti-MRP2/M2III-6 (Alexis Biochemicals, Grünberg, Germany) (Kool *et al*, 1997); monoclonal anti-MRP3/M3II-21 (Kamiya Biomedical, Seattle, WA) (Kool *et al*, 1997); monoclonal anti-MRP5/M5I-1 (Kamiya Biomedical) (Kool *et al*, 1997); goat-anti-rabbit and goat-anti-mouse IgG phosphatase labeled, NBT-BCIP Kit (Kirkegaard & Perry, Gaithersburg, MD); goat anti-mouse IgG fluorescein isothiocyanate conjugate and anti-rabbit IgG TRITC conjugate (Sigma); methanol, n-hexan (Baker, Deventer, the Netherlands). Other reagents were of highest grade commercially available.

Keratinocytes Normal human epidermal keratinocytes were obtained from foreskin by dispase (Boehringer Mannheim) separation of the epidermal sheet and subsequent trypsin/ethylenediamine tetraacetic acid digestion and the cells were cultured in low calcium (0.09 mM), serum-free, keratinocyte medium with bovine pituitary gland extract, recombinant human epidermal growth factor, insulin, gentamycin sulfate and amphotericin B as described by the manufacturer (Boehringer Mannheim). Cells were subcultivated by using the manufacturers detach kit with Hank's balanced salt solution and trypsin/ethylenediamine tetraacetic acid. The medium was replaced regularly three times a week. Cells were used for this study in the second and third passage in late subconfluency. Induction was made by the addition of inducers solved in dimethyl sulfoxide. The solvent concentration in the medium did not exceed 0.1%, controls were performed with dimethyl sulfoxide without inducers. Benz[*a*]anthracene (BA) was used in a concentration of 10^{-5} M over 2 d and dexamethasone as 10^{-7} M over 3 d.

RNA isolation mRNA was extracted from 5×10^6 cells from five different donors with the Oligotex Direct mRNA-purification kit (Qiagen, Hilden, Germany) using the mRNA-enrichment protocol (Kuribayashi *et al*, 1988). mRNA concentration of each sample was measured using the Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany) and similar amounts of mRNA were used for reverse transcription.

Reverse transcription–PCR Reverse transcription and PCR was performed with the GeneAmp RNA PCR kit (Perkin Elmer, Weiterstadt, Germany) according to the manufacturer's instructions. All reverse transcription–PCR experiments were performed in duplicate for each donor. Detection of specific mRNA for P450 enzymes and MDR1, MRP1–6 and LRP was achieved by using primers designed to amplify at least one intron in the gene to exclude contamination of cDNA with genomic DNA (**Table I**). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard as described before (Hakkola *et al*, 1994; Kool *et al*, 1997, 1999; Baron *et al*, 1998). Amplification was carried out with 35 cycles of 1 min denaturation at 93°C, 1 min annealing at 54°C and 1 min extension at 72°C. Amplification was terminated with an extension step of 5 min duration after the last cycle. PCR products were separated on 1.0% agarose gels (1 × Tris-buffered saline) and stained with ethidium bromide.

Cloning of PCR products into a PGEM-T vector PCR products were extracted from agarose gels using the QIAquick Gel Extraction Kit (Qiagen). PCR fragments were A-tailed and ligated into the pGEM-T vector using the PGEM-T Vector system (Promega, Mannheim, Germany). An aliquot of the ligation mix was transformed into *Escherichia coli* JM 109 competent cells (Promega) and plated on LB/amp/IPTG/X-gal plates. Bacteria were grown until colonies were visible and blue/white staining could be clearly distinguished. White colonies that generally contain inserts were picked and bacteria were grown in the presence of the antibiotic ampicillin. For plasmid preparation the QIAprep plasmid preparation system (Qiagen) was used.

Sequencing PCR product was sequenced after cloning. Sequencing was performed using the DYE Primer Cycle sequencing kit (Perkin Elmer) on an ABI Prism sequencer (Perkin Elmer). Homology searches in GenBank and EMBL database were done using the BLAST algorithm.

Immunoblot The microsome samples were prepared by homogenization of the cells through repeated sonification on ice in Tris/

Table I. Primers used for reverse transcription–PCR analysis

CYP	Sense primer location	Anti-sense primer location	PCR product
1A1	TCACAGACAGCCTGATTGAGA 928–947	GATGGGTTGACCCATAGCTT 1341–1360	433
1A2	TGGCTTCTACATCCCCAAGAAT 1199–1221	TTCATGGTCAGCCCGTAGAT 1488–1507	309
1B1	GTATATTGTTGAAGAGACAG 2423–2442	AAAGAGGTACAACATCACCT 2719–2738	316
2B6/7	CCATACACAGAGGCAGTCAT 1045–1064	GGTGTCAGATCGATGTCTTC 1402–1421	377
2E1	AGCACAACTCTGAGATATGG 925–944	ATAGTCACTGTACTTGAACT 1271–1290	366
3A4	CCAAGCTATGCTCTTCACCG 1279–1298	TCAGGCTCCACTTACGGTGC 1583–1602	324
3A5	TGTCCAGCAGAAACTGCAAA 1065–1084	TTGAAGAAGTCCTTGCGTGTC 1516–1535	471
3A7	CTATGATACTGTGCTACAGT 1041–1060	TCAGGCTCCACTTACGGTCT 1496–1515	475
MDR1	CCCATCATTGCAATAGCAGG 2712–2730	GTTCAAACTTCTGCTCCTGA 2849–2868	157
MRP1	TGGGACTGGAATGTCACG 241–259	AGGAATATGCCCCGACTTC 483–501	261
MRP2	CTGCCCTCTTCAGAATCTTAG 4072–4091	CCCAAGTTGCAGGCTGGCC 4294–4312	241
MRP3	CAGTCAGCCGCTCACCTATC 3458–3477	TCATCCAGTTCAGAGCAAAT 3747–3766	309
MRP4	CCATTGAAGATCTTCCTGG 12–30	GGTGTTCAATCTGTGTGC 243–250	239
MRP5	GGATAACTTCTCAGTGGG 336–353	GGAATGGCAATGCTCTAAAG 697–716	381
MRP6	CCATTGGGCTGTTTGCCTCC 3019–3038	GGTTGACCTCCAGGAGTCC 3237–3255	237
LRP	GTCTTCGGGCCTGAGCTGGTGTCG 1546–1569	CTTGGCCGTCTCTTGGGGGTCCTT 1762–1785	240
β-actin	ACCCACACTGTGCCCATCTA 488–507	CGGAACCGCTCATTGCC 761–777	290

KCl buffer pH 7.4 followed by differential ultracentrifugation. After 30 min centrifugation at 10,000 × *g* the supernatant was used for a subsequent ultracentrifugation for 90 min. The 100,000 × *g* pellet was suspended in Tris/KCl buffer. Protein was estimated according to Lowry *et al* (1951). The samples were boiled for 5 min in the presence of sodium dodecyl sulfate and β-mercaptoethanol according to Laemmli (1970). We loaded 50 μg microsomal protein into the lanes. Using 12% polyacrylamide gels with sodium dodecyl sulfate (Novex, Frankfurt, Germany), blotting was performed on to 0.45 μm cellulose nitrate sheets (Schleicher & Schuell, Dassel, Germany) according to the method of Towbin *et al* (1979) for 1 h with 200 V. Following blocking the remaining protein binding sites on the membrane with non fat dry milk powder in PBS, we incubated the blot in the primary antibody solution (each 1:1000 in PBS) overnight. After subsequent washing steps the blot was incubated in the secondary antibody solution (each 1:1000 in PBS) for 4 h. After subsequent washing steps the blot was developed with a phosphatase staining kit containing nitroblue tetrazolium and bromo-chloro-indoyl-phosphate for 5 min (Kirkegaard & Perry) to visualize the bands. Alternatively, the protein bands were detected on an autoradiography film using the enhanced chemiluminescence western blotting kit (Amersham, Little Chalfont, UK), according to the manufacturer's instructions.

Immunostaining CYP1A1-expression was demonstrated in differentiated keratinocyte multilayers cultured on sterile microscope slides (Nunc, Wiesbaden, Germany) for 48 h using an indirect immunoperoxidase technique. In order to detect intracellular CYP1A1, epitopes were unmasked using methanol and a target unmasking fluid (TUF; Kreatech Diagnostics, Amsterdam, the Netherlands) according to the manufacturer's instructions. Polyclonal antibodies specific for CYP1A1 (Oxygene, Dallas, TX) were applied (1:400) and detected by the APAAP method (Dako, Hamburg, Germany). After staining, slides were covered with Kayser's gelatine and photo-documentation was performed using a EP64 T Ektachrome film (Kodak, Stuttgart, Germany).

Immunofluorescence For immunofluorescent examination human foreskin specimen were fixed in 4% formaldehyde (Merck, Darmstadt, Germany) adjusted to pH 7.4 with sodium hydroxide for 24 h. After dehydration in alcohol, specimens were embedded in paraplast (Sherwood Medical, St Louis, MO) and cut into 8 μm sections with a microtome (Reichert-Jung, Nussloch, Germany). The sections were deparaffinated in Histoclear (Shandon, Frankfurt/Main, Germany), rehydrated in a declining alcohol concentration, washed in distilled water and prepared for antigen staining by microwave technique, using citrate buffer solution, pH 6.0, as a buffer for antigen retrieval (Evers and Uylings, 1994). Then the sections were hydrated in PBS for 10 min, incubated with 10% bovine serum albumin (Merck) for 30 min, and with the primary antibodies for another 45 min. After washing the slides three times with PBS, sections were incubated with secondary fluorochrome conjugated antibodies for 45 min, again washed with PBS, and mounted with Fluorprep (bioMerieux, Marcy l'Etoile, France). Following, the sections were stored at 4°C in the dark until examination with ultraviolet light and subsequent photodocumentation using Kodakchrome 400 ASA slide film (Kodak, Rochester, NY) and a photomicroscope (DMIL, Leitz, Wetzlar, Germany) equipped with epifluorescence illumination.

As primary antibodies we used polyclonal anti-serum for human CYP 1A1 and CYP 3A (Daiichi Pure Chemicals) and monoclonal antibodies to CYP 2B6, CYP 2E1 (Gentest), MDR1 (Signet Laboratories, Dedham, MA), and MRP1 (Chemicon) diluted 1:400 for the detection of the epitopes.

As a secondary antibody we used rabbit anti-mouse IgG (Fab-specific) fluorescein isothiocyanate conjugate (Dako) diluted 1:40 and rabbit anti-goat IgG (Fab-specific) CY3-conjugate (SIGMA) diluted 1:30.

Catalytic activities Freshly prepared microsomes from differential ultracentrifugation were immediately used for the catalytic assays. The protein determination was made according to Lowry *et al* (1951) using bovine serum albumin as standard. Assays were performed under dim red light. The amount of microsomal protein used was 0.1 mg per measurement (Jugert *et al*, 1994).

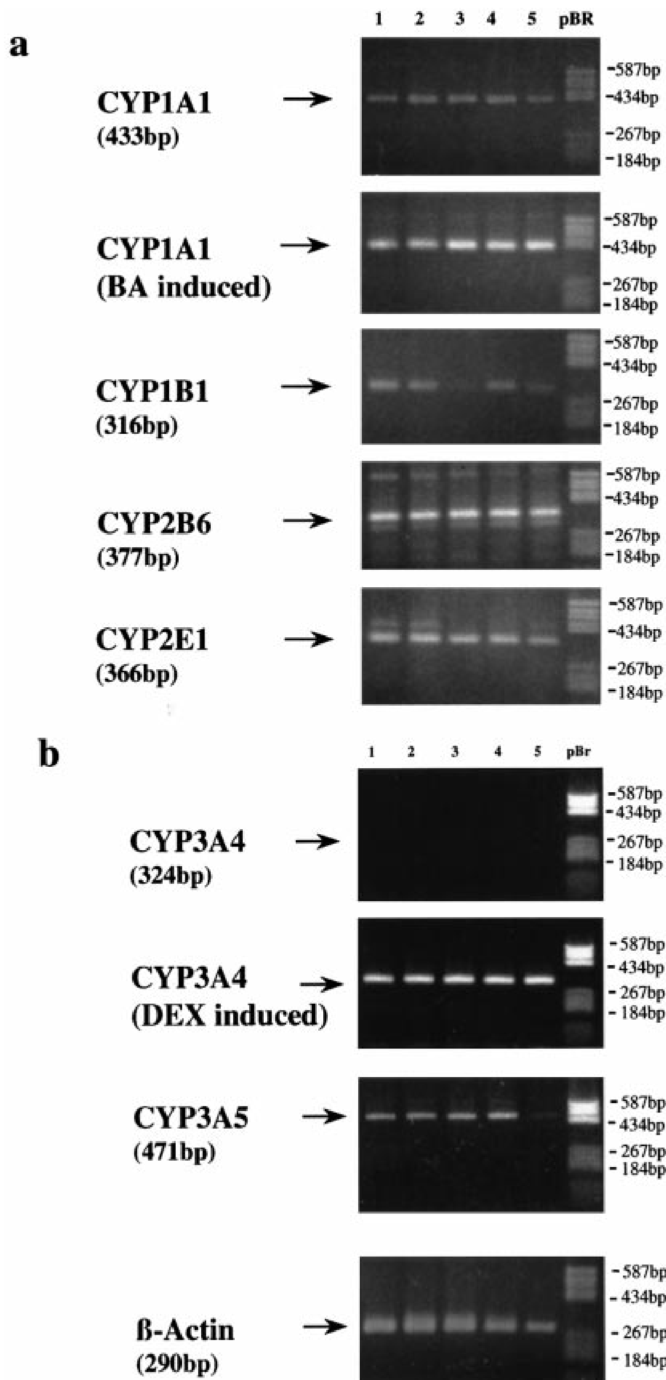

Figure 1. Reverse transcription–PCR of proliferating keratinocytes derived from the human epidermis of different donors with primers specific for various P450 enzymes and β-actin. Reverse transcription–PCR analysis of (*a*) CYP 1A1, 1B1, 2B6, 2E1, and (*b*) CYP3A4, 3A5, and β-actin expression. *Lane 1*, donor A; *lane 2*, donor B; *lane 3*, donor C; *lane 4*, donor D; *lane 5*, donor E; *lane 6*, DNA marker pBR322 *Hae*III digest. Induction of cells was performed with dexamethasone (10^{-7} M) or benzanthracene (10^{-6} M) for 24 h.

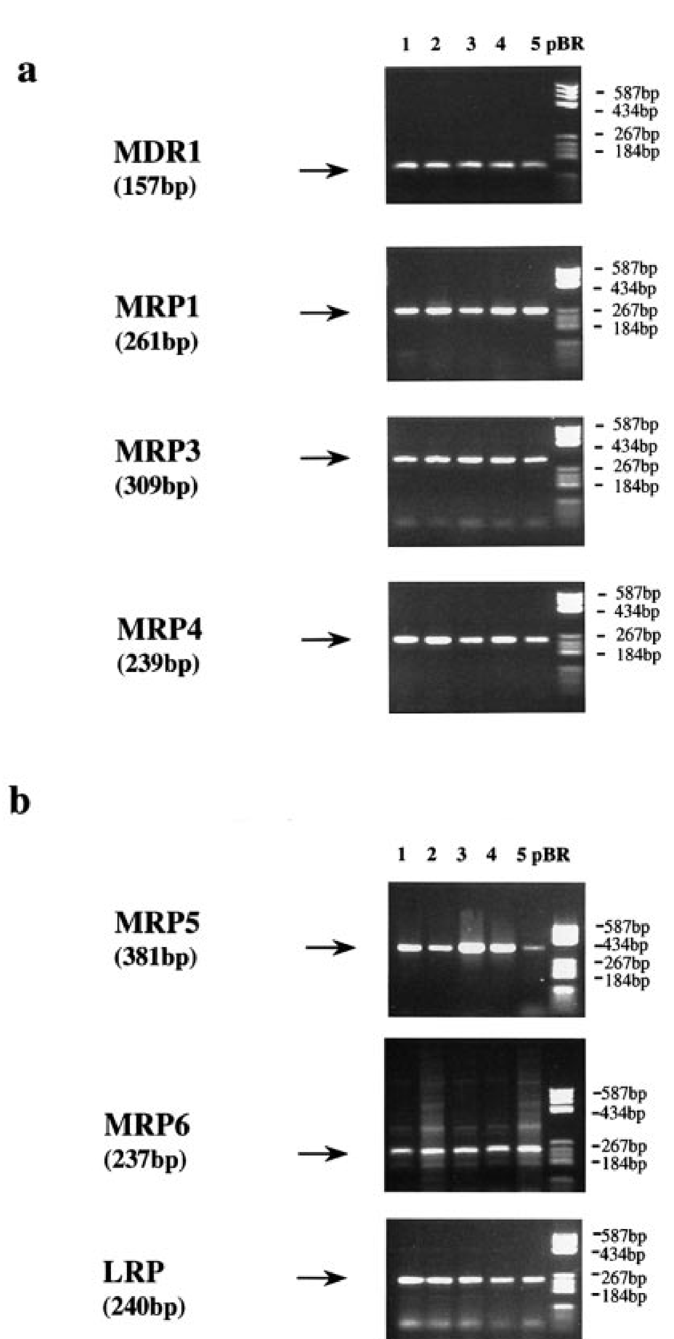

Figure 2. Reverse transcription–PCR of keratinocytes at different levels of subconfluency with primers specific for MDR1, MRP1–6, and LRP. Reverse transcription–PCR analysis of (*a*) MDR1, MRP1, MRP3, MRP4, and (*b*) MRP5, MRP6, and LRP expression. *Lanes 1–3*, keratinocytes 60% subconfluency (donor A); *lanes 4* and *5*, keratinocytes 90% subconfluency (donor A); *lane 6*, DNA marker pBR322 *Hae*III digest. Induction of cells was performed with dexamethasone (10^{-7} M) for 72 h.

Ethoxyresorufin O-Deethylase (Burke et al*, 1985)* The substrate 7-ethoxyresorufin is de-ethylated to resorufin in the presence of nicotinamide adenine dinucleotide phosphate and microsomes. The product resorufin is quantitated by a fluorocounter.

Pentoxyresorufin O-Deethylase (Lubet et al*, 1985)* The assay is the same as with ethoxyresorufin, but the substrate is 7-pentoxyresorufin.

ParaNitrophenol hydroxylase (Koop, 1986) In a CYP 2E1 specific reaction the paranitrophenol is hydroxylated in the presence of ascorbic acid, nicotinamide adenine dinucleotide phosphate and microsomes to 4-nitrocatechol, which can be quantitated by visible light.

Erythromycin N-demethylase Using this N-demethylation, which is specific for CYP3A isozymes, the amount of formaldehyde is determined by the Hantz'sch reaction (Nash, 1953; Jugert *et al*, 1994)

RESULTS

mRNA expression of CYP enzymes and MDR-associated proteins Expression of CYP1–3 gene families was studied by

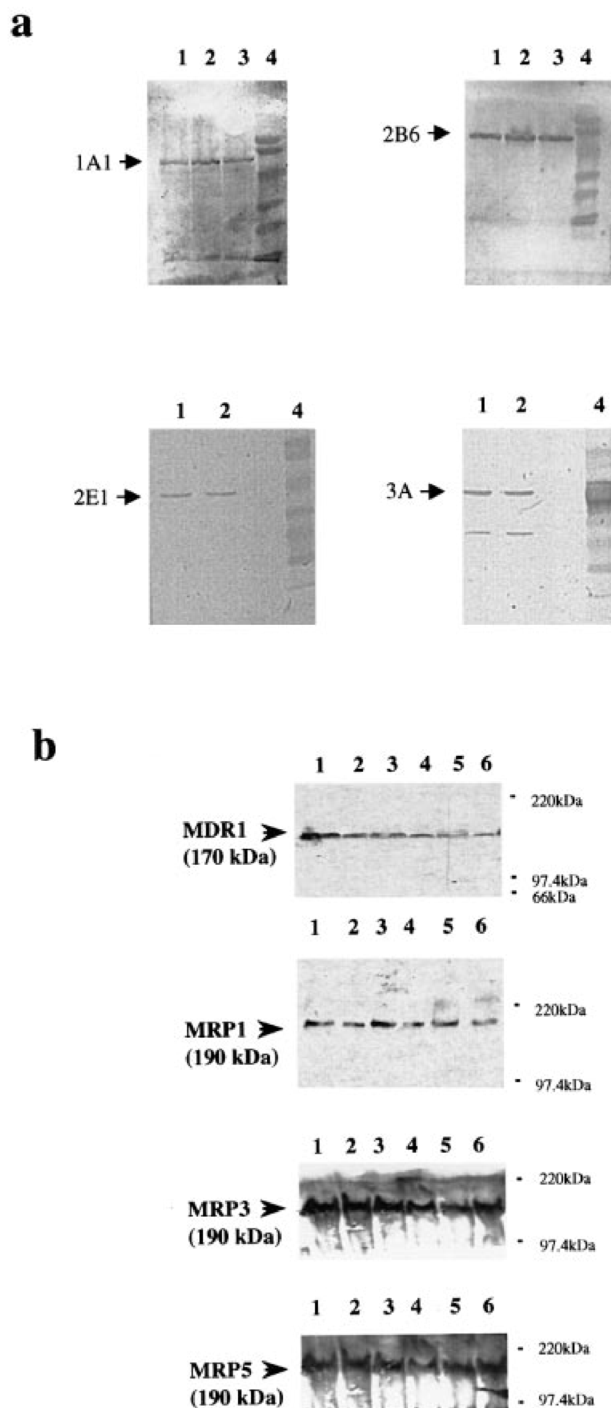

Figure 3. Immunoblots with antibodies against CYP enzymes and MDR-associated transport proteins. (*a*) Microsomal preparations from untreated proliferating human foreskin keratinocytes were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, blotted on to nitrocellulose sheets and subsequently treated with antibodies for cytochromes P450 1A1, 2B6, 2E1, and 3A5, respectively, and an alkaline phosphatase coupled secondary antibody. *Arrows* indicate the bands representing the particular constitutive cytochrome P450 enzyme. *Lanes 1–3*, donors A–C; *lane 4*, molecular weight marker: 106, 77, 50.8, 35.6, 28.1, 20.9 kDa. (*b*) The expression of MDR1, MRP1, MRP3, and MRP5 was detected via a 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis using specific monoclonal antibodies. Generally, 60 μg of protein are loaded per lane and only for the detection of MDR1 keratinocytes were induced with dexamethasone (10^{-7} M) for 72 h. The protein bands were detected on an autoradiography film using the enhanced chemiluminescence. *Lanes 1–6*, donors A–E (2×). Rainbow-colored protein molecular weight marker (myosin 220 kDa, phosphorylase b 97.4 kDa, bovine serum albumin 66 kDa).

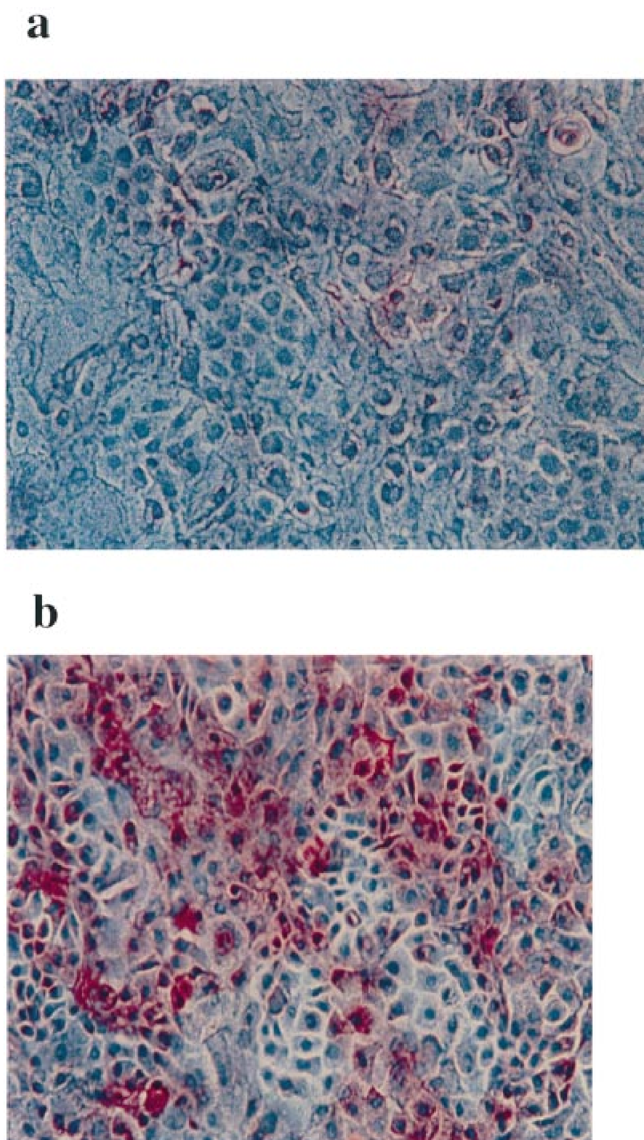

Figure 4. Expression of cytochrome P450 by intracellular staining in differentiated keratinocytes with an antibody against CYP 1A1. Intracellular immunohistochemical staining of differentiated normal epidermal keratinocytes after 48 h of Ca^{2+} preincubation and subsequent incubation with solvent (a) or (b) benzanthracene (2×10^{-5} M) for 72 h. After APAAP staining with an antibody against CYP1A1, (*a*) shows only a few positive cells, whereas in (*b*) an enhancement of CYP1A1 expression is shown.

reverse transcription–PCR in proliferating keratinocytes derived from the human epidermis of five male donors. Similar amounts of template mRNA, the same cycle no. (35) and annealing temperature were used for each reverse transcription–PCR reaction using different P450 primer pairs. For reverse transcription–PCR experiments (**Fig 1*a*, *b***) keratinocytes from the same degree of confluency and from the same pool of cells (donors A–E) were used for comparisons across different primer pairs. The results of those reverse transcription–PCR studies are displayed in **Fig 1**(***a***, ***b***). Keratinocytes revealed constitutive expression of CYP1A1, 1B1, 2B6, 2E1, and 3A5 and showed expression of CYP3A4 after incubation with dexamethasone (**Fig 1*b***). The expression of CYP1A1 was enhanced on the mRNA level after induction with BA (**Fig 1*a***).

Reverse transcription–PCR analysis of the MDR-associated transport proteins MDR1, MRP 1–6, and LRP revealed

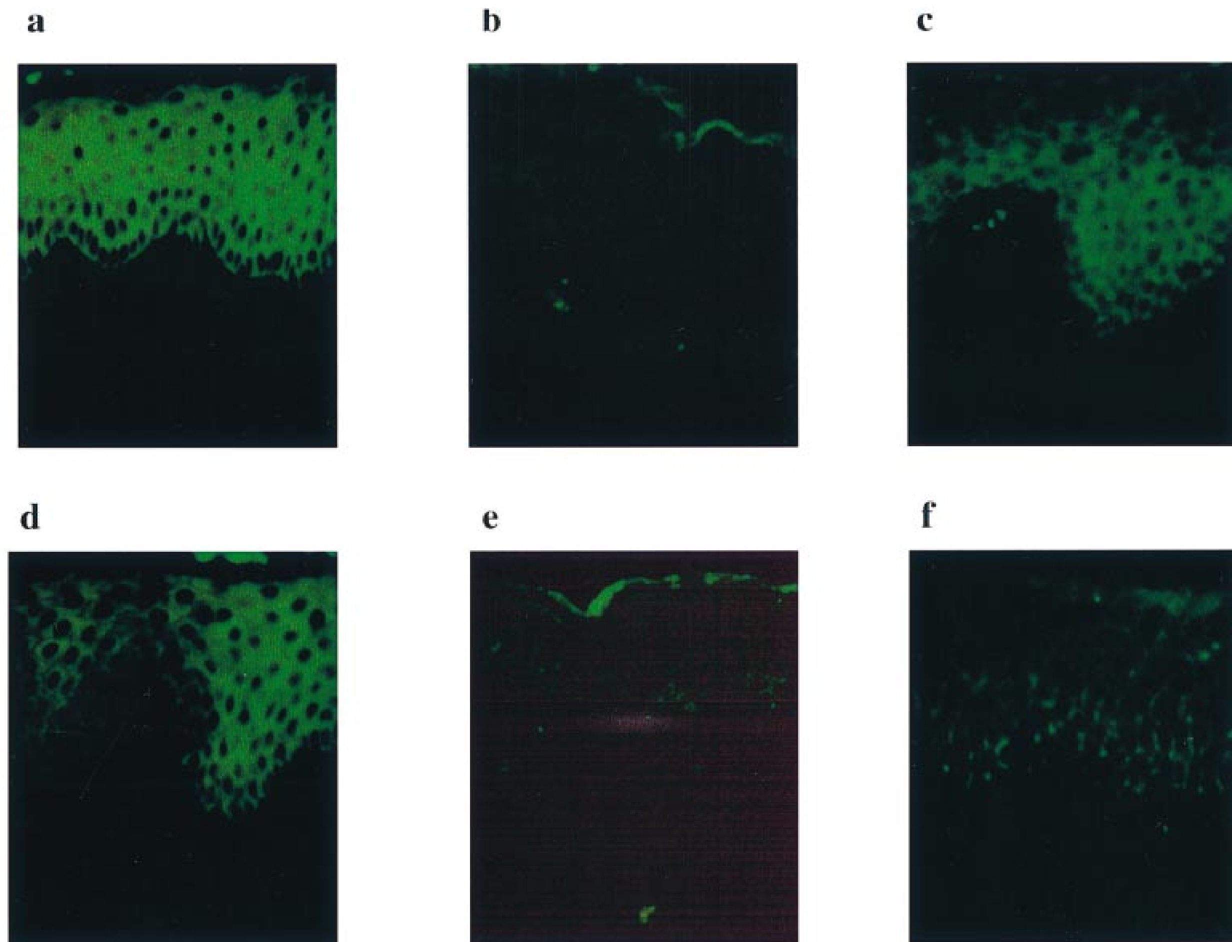

Figure 5. Expression of cytochrome P450 and MRP1 by intracellular staining in proliferating keratinocytes. Intracellular immunohistochemical staining of proliferating normal epidermal keratinocytes. Using antibodies against CYP 1A1 (*a*), 2B6 (*b*), 2E1 (*c*), 3A (*d*), MDR1 (*e*), and MRP1 (*f*) the expression of these proteins were shown by immunofluorescence.

constitutive expression of MRP1, MRP3, MRP4, MRP5, MRP6, and LRP in proliferating human epidermal keratinocytes (**Fig 2*a*, *b***) and was negative for MDR1 and MRP2 (not shown). Expression of MDR1 was seen after induction with dexamethasone. The level of subconfluency of those keratinocytes had no influence on the expression level of the transport proteins. As antibodies against some of the MDR-associated transport proteins analyzed in this study were not available, reverse transcription–PCR products from MDR1 and MRP1–6 were subcloned and sequenced (data not shown) to confirm their identity.

Protein expression of CYP enzymes and MDR-associated proteins Reverse transcription–PCR results were confirmed by immunoblots using specific antibodies against cytochrome P450 enzymes, which showed reactivity with CYP 1A1, 2B6, 2E1 and 3A (**Fig 3*a***). Further immunoblots using antibodies against MDR-associated transport proteins (**Fig 3*b***) revealed the expression of MRP1, MRP3, and MRP5, and was negative for MRP2 (not shown). MDR1 was detected in keratinocytes only after induction with dexamethasone (**Fig 3*b***). Specific antibodies against MRP4 and MRP6 were not available.

Immunohistochemistry After pretreatment of keratinocyte multilayers with "Target Unmasking Fluid" it was possible to show the expression of cytochrome P450 on the protein level by intracellular staining with specific antibodies using the APAAP method. In **Fig 4**(***a***) the antibody specific for CYP1A1 reveals the expression of this enzyme in cultured epidermal keratinocytes of the untreated control only in a few cells. After induction with BA the level of cytochrome P450 1A1 expression and the number of cells expressing CYP1A1 is highly increased (**Fig 4*b***). Using immunofluorescence we were able to identify CYP 1A1, 2B6, 2E1, 3A, MDR1, and MRP1 in keratinocytes of foreskin specimen. The localization of all CYP enzymes was restricted to the cytoplasm of the keratinocytes (**Fig 5*a–d***). A preferred staining was seen in the suprabasal layer of the epidermis. MDR1 was expressed in the cell membrane of the keratinocytes (**Fig 5*e***). MRP1 was demonstrated as membrane bound in keratinocytes (**Fig 5*f***).

Functional activity of CYP enzymes in human keratinocytes As shown in **Table II**, constitutive activity of several CYP enzymes were measured by catalytic assays. All of these activities can be enhanced by inducers, mediating their induction, e.g., by Ah-receptor (BA) or glucocorticoid responsiveness elements (DEX), known to increase the activities of CYP1A1 or 2B, 2E1, and 3A enzymes.

DISCUSSION

Interactions of xenobiotics, including drugs and tissues, such as the skin, are mainly influenced by metabolic capacities and transmembrane transport mechanisms. Synergism between both processes may occur in skin when metabolites produced by CYP enzymes, such as CYP3A, are better substrates for transport proteins, such as P-gp than the parent drug, or when P-gp prolongs the duration of absorption by necessitating the repeated entry of the drug into the keratinocyte. This process would increase exposure to CYP enzymes and could also prevent kinetic saturation of these proteins.

Table II. Microsomes (0.1 mg per tube) from human keratinocytes were used for catalytic assays[a]

	7-Ethoxy-resorufin-O-deethylase CYP1A	7-Pentoxy resorufin-O-deethylase CYP2B	Para-Nitro-Phenol-Hydroxylase CYP2E	Erythro-mycin-N-demethylase CYP3A
Basal activity	10.69	1.435	1.808	3.256
Induced activity	213.44	5.103	3.199	7.725
Inducer	20 mM benz[*a*]anthracen	4 mM phenobarbital	0.5 M ethanol	5 μM dexamethasone
Activity in min per mg protein	pmol resorufin	pmol resorufin	nmol 4-nitrocatechol	nmol formaldehyde

[a]Ethoxyresorufin is O-deethylated to resorufin by CYP1A enzymes. Pentoxyresorufin is O-deethylated to resorufin by CYP2B enzymes. ParaNitrophenol is hydroxylated to 4-nitrocatechol mainly by CYP2E1. Erythromycin is N-demethylated through CYP3A enzymes and the generated formaldehyde is determined by the Hantz'sch reaction.

Therefore, in this study the presence of these proteins was carefully studied on an RNA, protein, morphologic, and catalytic level. Reverse transcription–PCR, immunoblot, immunohistochemistry, and catalytic assays revealed that proliferating normal human skin keratinocytes show the expression of various CYP enzymes, especially CYP1A1, 1B1, 2B6, 2E1, and 3A. In comparison with other cells present in human skin, e.g., monocytes (Baron *et al*, 1998), lymphocytes (Brauers *et al*, 1998), and fibroblasts (not published) we measured considerably higher amounts of these enzymes in keratinocytes. By immunofluorescent techniques CYP 1A1, 2B6, 2E1, and 3A were demonstrated to be expressed dominantly in keratinocytes compared with fibroblasts (data not shown). In this study we showed the presence and inducibility of CYP1A1 on the mRNA level by reverse transcription–PCR (**Fig 1*a***) and on the protein level by immunohistochemistry (**Fig 4*a*, *b***). Inducer treatment resulted in a several-fold enhancement of ethoxyresorufin O-deethylase activity (**Table II**). Further experiments showed that phenobarbital increased the CYP2B catalyzed 7-pentoxyresorufin O-deethylase and dexamethasone induced erythromycin N-demethylase, which is preferentially mediated by CYP3A enzymes. Reverse transcription–PCR analysis confirmed these data and showed an induction of CYP3A4 after incubation with dexamethasone and a constitutive expression of CYP3A5. This is especially important because Wacher *et al* (1995) have shown that CYP3A is the primary CYP subfamily responsible for the phase I metabolism of over 50% of drugs administered to humans. These enzymes share a remarkable number of substrates with transport-associated proteins, such as P-gp. For a broad spectrum of these drugs, overlapping P-gp/CYP3A substrate specificities and tissue distributions present particular challenges to drug absorption and delivery to the systemic circulation. Significant decreases in bioavailability resulting from intestinal CYP3A-mediated metabolism of drugs, such as cyclosporine, are expected to be complemented by active extrusion of absorbed drugs through jejunal P-gp. Analogously, increased CYP and P-gp/MRP levels in skin tissues can possibly combine to diminish the effective tissue concentrations of these agents. Recent findings by Sleeman *et al* (2000) support this hypothesis. They were able to show that murine basal layer keratinocytes express functional phosphoglycoproteins and have the ability to expel the MDR-substrate rhodamine 123. Interestingly, in human keratinocytes the expression of polyspecific membrane transporters such as P-gp and of CYP enzymes, such as CYP3A4, was simultaneously upregulated after induction with dexamethasone (**Figs 1*b*, 2*a*, 3*b***).

Reverse transcription–PCR analysis and immunoblots also revealed a constitutive expression of MRP1, MRP3, MRP4, MRP5, MRP6, and LRP in human epidermal keratinocytes and were negative for MDR1 and MRP2. By immunofluorescent techniques the expression of MDR1 and MRP1 was demonstrated in the membrane of foreskin keratinocytes. MDR1 encodes a large glycosylated membrane protein, P-gp, that acts as an adenosine triphosphate-driven efflux pump. Based on its tissue distribution, P-gp has been proposed to play a part in the protection of the organism against xenobiotics. van Kalken *et al* (1993) have shown that cortisol is transported in an adenosine triphosphate-dependent process by P-gp expressing cells. Their results suggest a part for P-gp in steroid hormone transport, possibly to protect the cellular membranes from potentially toxic concentrations of steroids.

Although increased levels of P-gp are likely to contribute to MDR, it has become evident that alternative, non-P-gp-mediated mechanisms of MDR exist. Further studies have discovered the existence of other transport-associated proteins, such as MRP1 and MRP2. Both proteins are involved in the transmembrane transport of glutathione S-conjugates, which is essential for the elimination of such conjugates from the cell and it was shown in this study that at least MRP1 is expressed in keratinocytes. From the presence of MRP in many epithelia lining external surfaces, Flens *et al* (1996) suggested that MRP1, like P-gp, may have an excretory function in protecting the organism against xenobiotics. A search of the human Expressed Sequence Tag database by Kool *et al* (1997) found four transporters related to MRP1 and MRP2 and analyzed the expression of these genes, called MRP3–6, in normal tissues and tumor cell lines and all of them were detected in keratinocytes as shown. Recent studies revealed that MRP3 is an organic anion and multidrug transporter that confers high-level resistance to methotrexate but also has a possible physiologic role as a bile salt transporter in the small intestine. Other studies showed that mutations in the MRP6 gene cause pseudoxanthoma elasticum, a connective tissue disorder affecting elastic structures in the body including the skin, although the exact pathomechanisms of pseudoxanthoma elasticum are not completely understood (Ringpfeil *et al*, 2000).

The LRP, which was recently identified as the major vault protein, is present at elevated levels in many non-P-gp MDR cell lines and, as shown here, in keratinocytes. Vaults are located in the cytoplasm and it is speculated that they are part of a bidirectional transport system between nucleus and cytoplasm. Izquierdo *et al* (1996) have raised the possibility that vaults are involved in the transport of drugs into cytoplasmic vesicles or directly out of the cell. The ubiquitous presence of LRP in a broad panel of highly specialized cell types, including human keratinocytes suggests that vaults fulfil a common basic function in all cells, although cell type-specific roles cannot be excluded. The ongoing functional characterization of vaults is difficult, because complex structures such as complete vaults cannot be simply overproduced by transfection even if all the subunits have been cloned (Borst *et al*, 1997).

This study revealed that human epidermal keratinocytes express various metabolic active enzymes and transport-associated enzymes and are, therefore, capable of metabolizing and eliminating different xenobiotics. Especially the discovery of the expression of ATP-binding cassette transporters, such as MRP 1, 3, 4, 5, and 6 whose physiologic function has not been completely understood yet, seems to be a complete new factor in the structure of an active human skin barrier. Further studies have to characterize the physiologic role of these proteins in keratinocytes in order to find

out if biotransformation and anti-transport do act synergistically as a drug bioavailability barrier in human skin.

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