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# Expression of P450 enzymes in rat whole skin and cultured epidermal keratinocytes

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**Abstract**

The complement and level of expression of P450 enzymes in male Fischer F344 rat whole skin and cultured keratinocytes were investigated using a panel of mono-specific antibodies. In whole skin microsomal fraction, immunoreactive bands corresponding to CYP2B12, CYP2C13, CYP2D1, CYP2D4, CYP2E1, CYP3A1, and CYP3A2 were detected whereas CYP1A1, CYP1A2, and CYP2C12 were absent. Skin levels were all between 0.1% and 4.7% of those found in liver, except for CYP2D4, which was not detected in liver. Keratinocytes were isolated from rat skin, cultured for up to 42 days, and changes in the levels of CYP3A1, CYP3A2, and CYP2E1 determined. Levels were low in isolated keratinocytes, but they increased markedly in culture, reaching a maximum at 10–14 days, where they were similar to those found in fresh skin. This suggests that primary keratinocytes grown in culture for 10–14 days may provide a useful experimental model to study P450-catalysed metabolism of xenobiotics in skin. 

*Keywords:* Cytochrome P450; CYP; Rat; Skin; Cell culture; Keratinocytes

The skin provides an important route by which xenobiotics can enter the systemic circulation and since this tissue is metabolically active, such chemicals may be activated or detoxified by cutaneous enzymes during absorption. Such biotransformations occur mostly in the living cells of the epidermis and are due to both phase I and II reactions, with cytochrome P450 (P450; CYP) enzymes catalysing the majority of the phase I reactions [1]. The P450 enzymes, which comprise the products of a multigene superfamily, can catalyse the metabolic activation of both xenobiotics and endobiotics [2]. They are of special interest in the development of pharmaceuticals for skin diseases because many drugs used to treat dermatological diseases are substrates, inducers or inhibitors of P450 enzymes. A detailed insight into the expression and activity of cutaneous P450 enzymes, that are capable of modulating drug bioavailability and skin sensitisation through chemical activation, will be helpful in the development of improved strategies for the therapy of skin disorders.

The majority of P450 enzymes responsible for the biotransformation of xenobiotics belong to families CYP1, CYP2, and CYP3. They are expressed predominantly in the liver. In the rat, many of these P450 enzymes have also been shown to be expressed in extrahepatic tissues [3–5] and some in skin [6–8]. The cutaneous expression of CYP1A1 has been studied extensively because of its role in procarcinogen biotransformation [9–11]. However, relatively little is known about the expression of other P450 enzymes in rat skin.

Of particular interest is the expression of P450 enzymes in epidermal keratinocytes. These cells make up about 95% of the cell mass of human epidermis and are responsible for the biochemical and physical integrity of skin. Cultured primary keratinocytes and established keratinocyte cell lines are increasingly used to study the cytotoxicity of topically applied compounds and preparations. However, the metabolic capabilities of these cells, in well-defined culture conditions, are largely undefined. We have developed a panel of antibodies that

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bind specifically to each of the major xenobiotic metabolising P450 enzymes in rat [12] and these have been used to identify P450 enzymes expressed in liver and extra-hepatic tissues, e.g., [5,13]. Therefore, these antibodies can be used to identify the P450 enzymes expressed in rat skin.

Hence, the aim of this study was to investigate the profile of P450 enzymes expressed in rat skin and to determine the level of expression of P450 enzymes in cultured primary keratinocytes.

## Materials and methods

*Materials*. Keratinocyte serum-free medium, Hanks' balanced salt solution (HBSS), gentamicin, fungizone, penicillin–streptomycin, trypsin–EDTA, and foetal calf serum were obtained from GIBCO (Life Technologies, Paisley, UK). All other chemicals were purchased from Sigma (Poole, UK).

*Preparation of rat skin*. Male Fischer F344 rats weighting about 200 g were killed by an appropriate humane procedure, their hair was shaved, and the dorsal region of the skin was removed. Full-thickness skin was prepared as described previously [14], and then cut into small pieces in preparation for isolation of keratinocytes or skin microsomal fraction.

*Freshly isolated and cultured epidermal keratinocytes*. Rat skin prepared as described above was cleaned in HBSS supplemented with 20 μg/ml gentamicin and digested with 0.2% (w/v) protease in HBSS containing gentamicin for 20–22 h at 4 °C. On the following day, the epidermis was separated from dermis and the epidermal preparation was placed into trypsin–EDTA solution (0.5 g/L trypsin and 0.2 g/L EDTA) for 2 h at room temperature. Cells were dissociated by repeated aspiration, filtered through a sterile nylon gauze (0.25 μm pore size) to remove any hair or stratum corneum, and resuspended in complete keratinocyte medium (keratinocyte serum-free medium, which contains 25 μg/ml bovine pituitary extract and 0.2 mg/ml epidermal growth factor, supplemented with 10% foetal calf serum, 2.5 μg/ml fungizone, 1000 U/ml penicillin, and 100 μg/ml streptomycin). The cells were sedimented twice by centrifugation at 100*g* for 5 min and resuspended each time in complete keratinocyte medium. Cell number and viability were assessed by trypan blue exclusion. The cells were cultured by seeding them into T75 flasks at a density of approximately $6 \times 10^6$ cells per flask in 15 ml complete keratinocyte medium. The flasks were incubated at 37 °C for 18 h in an atmosphere of 5% $CO_2$/95% air. Following this, the medium was replaced with keratinocyte serum-free medium containing 2.5 μg/ml fungizone, 1000 U/ml penicillin, and 100 μg/ml streptomycin and, subsequently, this medium was changed every 2–3 days.

*Preparation of microsomal fractions from skin, cultured keratinocytes, and liver*. Full-thickness skin from four male rats was prepared as described above and then minced in ice-cold phosphate-buffered saline (pH 7.4). Homogenisation was performed with an Ultra-Turrax T25, probe S25N-10G (Janke & Kunkel, IKA, Labortechnie, Germany) using $4 \times 20$ s bursts with 20 s cooling periods between each burst. The homogenate was filtered through two layers of nylon mesh to remove any hair or other debris and immediately subjected to differential centrifugation to prepare the microsomal fraction as described previously [15].

Keratinocytes cultured as described above were harvested by scraping into 10 ml ice-cold phosphate-buffered saline, pH 7.4 (PBS), and sedimented by centrifugation for 5 min at 100 g. After three cycles of washing in this buffer, the sedimented keratinocytes were resuspended in 0.5 ml PBS and disrupted for 2 min in a sonicating water bath. The microsomal fraction of the lysate was then prepared by differential centrifugation [15].

Liver microsomal fractions were prepared from untreated male and female rats and male rats treated with 3-methylcholanthrene (MC), phenobarbitone (PB), or pregnenolone 16α-carbonitrile (PCN) as described previously [16]. Liver microsomal fractions from treated rats contained elevated levels of CYP1A1 and CYP1A2 (MC), CYP2B1, and CYP2B2 (PB), and CYP3A1 and CYP3A2 (PCN), amongst other P450 enzymes.

The protein content of all samples was determined by the method described by Lowry et al. [17] using bovine serum albumin fraction V as standard.

*Immunoblotting analysis*. Imunoblotting of skin, keratinocyte, and liver microsomal fractions was performed as described previously [15] using a panel of anti-peptide antibodies targeted against rat P450 enzymes, the properties of which have been described previously [5,12,18]. Immunoblots were developed with each of the antisera diluted as follows: anti-CYP1A1 (1:4000), anti-CYP1A2 (1:4000), anti-CYP2B1/2 (1:2000), anti-CYP2E1 (1:4000), anti-CYP2C12 (1:4000), anti-CYP2C13 (1:4000), anti-CYP2D1 (1:4000), anti-CYP2D4 (1:4000), anti-CYP3A1 (1:8000), and anti-CYP3A2 (1:8000). Immunoreactive band intensity was quantified using a Kodak Image Station and Kodak Digital Science 1D Image Analysis Software (NEN Life Science Products, Hounslow, UK).

## Results

### *Expression of P450 enzymes in whole skin*

To identify the P450 enzymes expressed in normal rat skin microsomal fraction, immunoblotting was performed using anti-peptide antibodies targeted against a panel of rat P450 enzymes. The antibodies targeted against CYP2B1/2, CYP2C13, CYP2D1, CYP2D4, CYP2E1, CYP3A1, and CYP3A2 all produced single immunoreactive bands in rat skin microsomal fraction (Fig. 1). The migration of each of these bands corresponded to those of the target P450 enzyme in liver microsomal fraction from either untreated or appropriately induced rats, except for the antibody against CYP2D4, which produced a single immunoreactive band of approximately 52 kDa in skin microsomal fraction, although no immunoreactive bands were detected in the liver, and the antibody against CYP2B1/2, which produced a band in skin microsomal fraction that migrated slightly faster than that in liver (Fig. 1). With the exception of CYP2D4, the levels of all of these P450 enzymes were lower in skin compared with liver. The relative intensity of immunoreactivity in samples of skin microsomal fraction compared with liver microsomal fraction from untreated rats was 2.3% for CYP2B1/2, 0.3% for CYP2E1, 1.3% for CYP2C13, 0.1% for CYP2D1, 4.7% for CYP3A1, and 0.4% for CYP3A2 (all values are means of three determinations).

No immunoreactive bands were detected in skin microsomal fraction using antibodies targeted against CYP1A1, CYP1A2 or CYP2C12, even though immunoreactive bands corresponding to these P450 enzymes were clearly detected in samples of liver microsomal fraction from an untreated rat (which contains CYP1A2

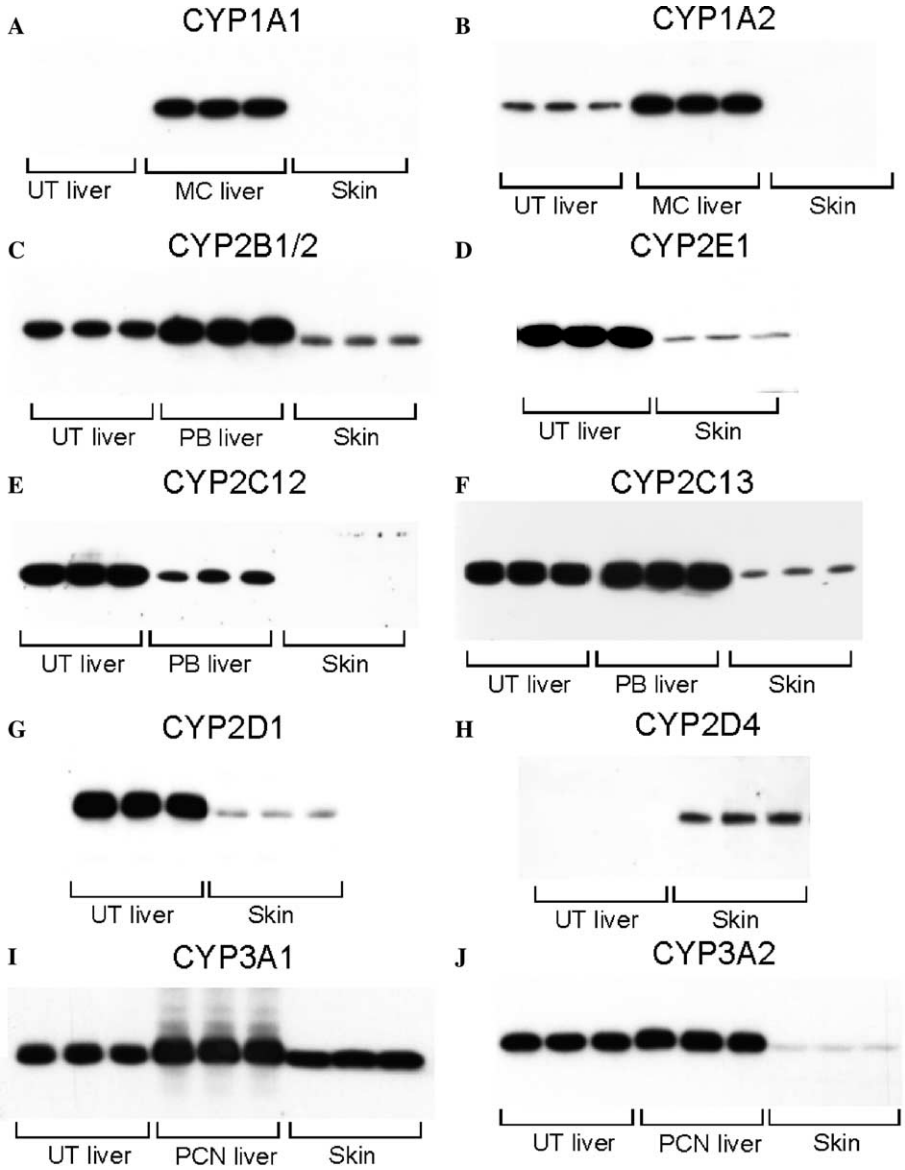


Fig. 1. Expression of P450 enzymes in rat full-thickness skin microsomal fraction. Samples of microsomal fraction from rat liver and skin were separated by SDS–PAGE and then transferred onto nitrocellulose filters. Each of the nitrocellulose filters was developed with an anti-peptide antibody targeted against (A) CYP1A1, (B) CYP1A2, (C) CYP2B1/2, (D) CYP2E1, (E) CYP2C12, (F) CYP2C13, (G) CYP2D1, (H) CYP2D4, (I) CYP3A1, or (J) CYP3A2 as described in Materials and methods. All samples were from male rats, except in blot (E) where the untreated liver sample was from female rats. For the liver samples, 1 μg protein was loaded, except for (D) and (G) where 0.2 μg was applied, and for the skin samples 20 μg protein was loaded in all cases. Each sample was loaded three times. Only the central section of each blot is shown in the region of 45–60 kDa.

and CYP2C12) or an MC-treated rat (which contains high levels of CYP1A1 and CYP1A2).

*Keratinocyte culture*

Keratinocytes were cultured in serum-free medium for up to 42 days (Fig. 2). It was found that the cells attached themselves to the surface within 24 h of seeding and then grew to form a monolayer of proliferative cells. These dividing cells reached near confluence by 10–14 days and maintained an even monolayer until 28 days. Thereafter, the integrity of the culture began to deteriorate; cells began to grow on the top of the monolayer and those at the edges progressively took on a sparse appearance. By 35–42 days, all of the cells had become rounded, small, and non-proliferative. They appeared loosely packed, although still attached on the surface of the culture flask.

*Expression of P450 enzymes in cultured keratinocytes*

Changes in the relative levels of expression of CYP3A1, CYP3A2, and CYP2E1 were determined in the cultured keratinocytes over time. On day 0, it was found that all 3

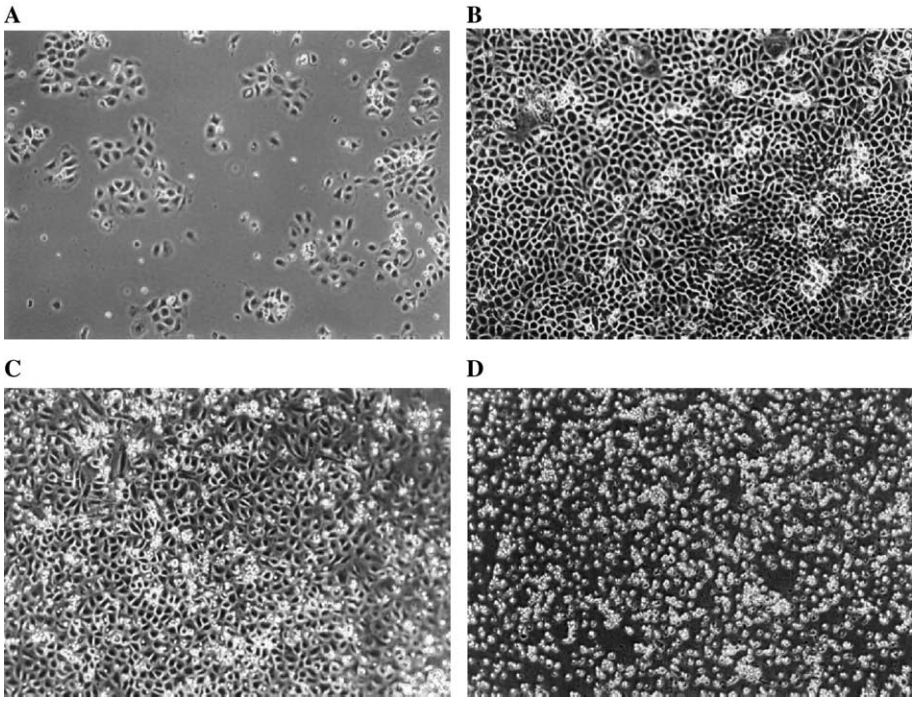


Fig. 2. Changes in the morphology of primary keratinocytes during culture. Keratinocytes were prepared from rat skin and then placed in culture in serum-free keratinocyte medium, as described in Materials and methods. Photographs of the cultures were taken at 250× magnification at (A) 3 days, (B) 14 days, (C) 21 days, and (D) 42 days after seeding.

proteins were poorly expressed, with CYP2E1 being undetectable (Fig. 3). However, the levels increased progressively in culture between 0 and 14 days (Fig. 3). Maximum expression of CYP3A1, CYP3A2, and CYP2E1 was observed at 10–14 days, where levels peaked at 98%, 104%, and 137% of the respective levels in skin microsomal fraction (Fig. 3). This coincided with the time when the cells approached confluence (Fig. 2). Thereafter, as the integrity of the culture decreased, the levels of the P450 proteins also declined progressively. After 42 days in culture, only a low level of CYP3A1 was detected whilst CYP3A2 and CYP2E1 levels were below detection.

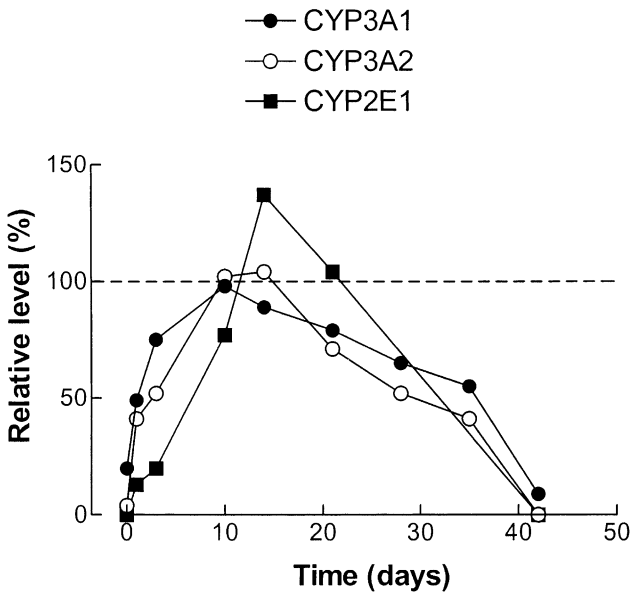


Fig. 3. Variation in the expression of P450 proteins in rat keratinocytes over time in culture. Rat keratinocytes were prepared and cultured for up to 42 days, under the conditions described in Materials and methods. At intervals, the cells were harvested, the microsomal fraction was prepared, and immunoblotting was performed in order to determine the levels of CYP3A1 (●), CYP3A2 (○), and CYP2E1 (■) relative to those expressed in full-thickness skin microsomal fraction obtained from a pool of four rats. Each value is the mean of duplicate determinations and the data shown are representative of three experiments for CYP3A1 and CYP3A2 and two experiments for CYP2E1, which produced similar results on each occasion.

## Discussion

In this study, we have investigated the expression of P450 enzymes in rat skin by immunoblotting with a panel of anti-peptide antibodies. Anti-peptide antibodies were directed towards small defined regions of each of the respective P450 enzymes. The specificity of such antibodies has been demonstrated in previous studies that have shown that the antibodies bind only to those P450 enzymes containing the target epitope [12,19]. In practice, this usually means that each of the antibodies recognises a single P450 enzyme. Binding to more than one P450 enzyme occurs only if the identical (or extremely similar) target peptide region is present in other P450 enzymes [12]. This is likely to occur only in P450

enzymes from the same subfamily, as it is only these proteins that share sufficient sequence similarity. Consequently, identification of single immunoreactive bands of 50–54 kDa by immunoblotting of skin microsomes using antibodies targeted against CYP2B1/2, CYP2C13, CYP2D1, CYP2D4, CYP2E1, CYP3A1, and CYP3A2 strongly indicates the presence of these, or highly related, P450 enzymes in rat skin. Also, the lack of immunoreactive bands using antibodies against CYP1A1, CYP1A2, and CYP2C12 indicates the absence of these forms of P450 in rat skin.

The immunoreactive band detected in skin with the antibody originally targeted against CYP2B1/2 is likely to be CYP2B12. The epitope to which this antibody was directed in CYP2B1 and CYP2B2 (residues 265–276 of CYP2B1/2: Ile–Asp–Thr–Tyr–Leu–Leu–Arg–Met–Glu–Lys–Glu–Lys) is identical in CYP2B12 [7] and therefore the antibody is expected to bind as well to CYP2B12 as it does to both CYP2B1 and CYP2B2. Also, the immunoreactive band in skin was found to migrate slightly faster than that in liver, an observation also reported by Pham et al. [6] who used a polyclonal antibody raised against purified CYP2B1. This would be consistent with the relative migration of CYP2B12 in comparison with CYP2B1 [7] present in skin and liver, respectively.

CYP2E1 has not been identified previously in rat skin, although immunoreactive bands corresponding to CYP2E1 and a low level of *p*-nitrophenol hydroxylation have been detected in mouse skin, particularly after treatment with dexamethasone [20,21]. It has been known for some time that rat skin microsomal fraction is active in the oxidation of testosterone to 6β-, 7α-, and 16α-hydroxylated products through P450-catalysed reactions [22], suggesting the presence of CYP3A enzyme(s). However, no previous studies have identified specific CYP3A enzymes in rat skin, although immunoreactive bands were detected in mouse skin [20]. Here, it was found that both CYP3A1 and CYP3A2 are expressed in rat skin. Interestingly, CYP3A1 was previously shown to be expressed in liver, kidney, lung, and small intestine, whereas CYP3A2 was found only in the liver, although expression in skin was not investigated [5]. In addition to these P450 enzymes, skin microsomal fraction was found to contain CYP2C13, CYP2D1 and CYP2D4. The expression of none of these P450 enzymes in skin appears to have been described previously. Altogether, the expression of a variety of P450 enzymes in rat skin suggests that this organ has the capacity to metabolise a wide range of xenobiotics and endogenous substrates. However, the levels of the P450 enzymes were low (<5%) compared with those expressed in liver; consequently, P450-catalysed metabolism will occur only at a relatively slow rate.

The absence of CYP1A1 from the skin of untreated rats is in agreement with previous studies that have shown that this P450 enzyme is expressed in skin only after treatment with polycyclic aromatic hydrocarbons [6,9,23]. Previously, CYP1A2 has been shown to be expressed in the liver, but not extra-hepatic tissues [4]. The lack of CYP1A2 in the skin is consistent with this observation. Also, no CYP2C12 was detected in rat skin. This enzyme is normally expressed in female and not male liver [24]. Here, as expression was examined only in male skin, it remains possible that CYP2C12 is expressed in female skin.

In contrast to whole skin, freshly isolated keratinocytes were deficient in P450 enzymes, as amongst the three P450 enzymes examined only CYP3A1 was detected and even this was at a very low level. It seems likely that this is due to the protracted preparative procedure, which takes about two days to complete, although the mechanism of loss of expression is not known. Clearly, in view of this, the usefulness of freshly isolated epidermal cells for any in vitro studies that rely on P450-catalysed metabolism is questionable.

The levels CYP3A1, CYP3A2, and CYP2E1 gradually recovered during culture of the keratinocytes over 2 weeks to levels similar to those of fresh whole skin. The reason for the re-expression of the P450 enzymes is unknown, but may be related to the proliferative activity of the keratinocytes. It would be of interest to determine not only the range of P450 enzymes expressed after culture, but also the mechanism that controls P450 gene expression under these conditions. In any case, these preliminary data suggest that 10–14-day cultured keratinocytes express P450 enzymes at levels close to those of whole skin and so may provide an excellent model for investigating the metabolism and toxicity of drugs and other xenobiotics in the skin. Although only primary cell cultures were examined here, it is also possible that the keratinocytes may be passaged a limited number of times to expand cell number and perform a greater number of experiments. Preliminary work by Chun et al. [25] suggests that this may be possible. However, such cells need to be more thoroughly characterised to help assess their potential utility.

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