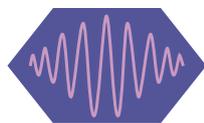


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Skin Metabolism Focus Group Meeting

Charles River, Edinburgh, UK - 20 May 2011

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AGENDA

We are pleased to welcome you to the Skin Metabolism Skin Forum focus group meeting. The meeting will be divided into a morning lecture session followed by an afternoon breakout discussion forum session. There will be a poster session.

Speakers

Simon Wilkinson - Newcastle University, UK
Cutaneous Metabolism – A Historical Perspective

Christine Jäckh - BASF, Germany
Skin Equivalents – How Do They Contribute to Skin Metabolism and Differ to *ex vivo* Skin?

Joan Eilstein - L'Oreal, France
Comparison of Xenobiotic Metabolizing Enzyme Activities in Normal Human Skin and Reconstructed Human Skin Models from SkinEthic Laboratories

Christine Götz - University of Düsseldorf, Germany
Xenobiotic Metabolism in Human Skin and *in vitro* Alternatives: Phase I & II

Raniero Zazzeroni - Unilever, UK
Drivers for Assessing Metabolism of Chemicals in Skin – Safety Assessment for Skin Sensitisation Without Animal Testing

Chair

Clive Roper - Charles River, UK

Proposed Breakout Topics

Are we doing it right?

How do we answer qualitative questions (is my compound metabolised or not)?

How do we answer quantitative questions?

How do we assess metabolic “competency” and positive controls?

3D models and their utility

How useful/applicable are *in vitro* animal models in predicting human metabolism?

What is the future? What do we need to increase our confidence in the data?

Moderators

Clive Roper and **Simon Wilkinson**



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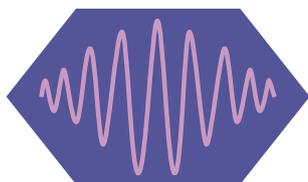


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Cutaneous Metabolism – A Historical Perspective

Simon Wilkinson and Faith Williams, Medical Toxicology Centre, Institute for Cellular Medicine, Newcastle University, UK

The activity of metabolic enzyme systems in the skin, and the importance of cutaneous metabolism of exogenous xenobiotics in disease causation, have been known for many years. From the late 70s onwards, our knowledge of the specific enzyme systems involved in cutaneous Phase I and Phase II metabolism increased markedly. Expression (mRNA and protein) and catalytic activity (typically to classical probe substrates) of a range of enzyme systems were reported in a number of cutaneous tissues, including fresh human and laboratory animal skin (intact and in tissue homogenates), primary keratinocytes and (for selected enzymes) immortalized cell lines. These enzymes included numerous isoforms of the CYP family (especially 1A1/2, 1B, 2A, 2E1, 3A4/5 and others), carboxylesterases, other esterase classes, oxidoreductases (alcohol and aldehyde dehydrogenases, NADP(H) quinone oxidoreductase) and the full range of transferase enzymes (sulpho-, glucuronyl, glutathione, and N-acetyltransferases). Generally, levels of expression and activity per unit weight of tissue were much lower than the corresponding liver enzymes (apart from NADP(H) quinone oxidoreductase). However, histochemical and/or mRNA expression studies showed that many of these enzyme systems were expressed in specific cell types (basal epidermis, hair follicles and sebaceous glands). The presence of different isoforms of certain enzymes from those expressed in liver was identified. Important species differences were also recognised, suggesting that laboratory rodent models were not necessarily representative of human skin. More recently, the control of enzyme activity (especially CYP isoforms under the control of AhR and ARNT) has been further elucidated. A novel CYP isoform, CYP2S1, has been identified, which appears to be more highly expressed in skin than in liver. In the last ten years, there has been a renewed interest in the role of cutaneous metabolism in toxicity (especially skin sensitisation, driven by upcoming legislation) and transdermal drug delivery. A number of reports of functional studies of metabolism of ethoxycoumarin, benzo[a]pyrene and bisphenol A during percutaneous absorption through pig ear skin have been published which suggest that porcine ear skin may be an effective model for human metabolism during percutaneous absorption, though further comparative investigation is needed. Similar studies on esterase activity in human and minipig skin using parabens as substrates have suggested the presence of two isoforms (hCE1 and hCE2) in human skin, with differing substrate specificity for the ester groups of parabens. In parallel with studies on ex vivo skin, a growing number of reports of metabolic activity and mRNA/protein expression of phase I and II enzymes in human skin equivalents (or reconstructed epidermis) have been published. These reports also indicate the potential utility of reconstructed epidermal and full thickness models for assessment of cutaneous metabolic contributions to dermal disposition and activity. Our understanding of cutaneous metabolism of endogenous substrates such as retinoic acid, androgens/oestrogens and melanin, as well as the enzymology of corneocyte desquamation, has also increased considerably in recent years. A number of challenges remain, however. A comprehensive comparison of isoforms of phase I and phase II enzymes between human and porcine skin should be carried out. The relationship between absorption/penetration rate and metabolism remains to be fully elucidated. The potential for in situ methods such as microdialysis for measurement of cutaneous metabolism has not yet been fully explored.

Skin Equivalents – How Do They Contribute to Skin Metabolism and Differ to *ex vivo* Skin?

Christine Jäckh¹, Veronika Blatz¹, Kerstin Reisinger², Eric Fabian¹, Bennard van Ravenzwaay¹, Robert Landsiedel¹

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Reconstructed human skin equivalents (RHE) are considered a useful *in vitro* system to examine toxic effects of substances to the skin. One step leading to skin toxicity is metabolic activation by the xenobiotic metabolizing system. Investigations on the metabolic competence of RHE however remained neglected and often gene expression profiles rather than actual enzyme activities are reportedⁱ.

In A German BMBF-funded project, enzymatic activities in human skin equivalents were measured and compared to native human skin. It shows that in respect to metabolic activity complex reconstructed full-thickness skin models are closer to native human skin than simple cell culture models. It also shows that some enzymes such as CYP1A are expressed in the RHE, but do not exhibit detectable activity. On the other hand expression profiles helped to assign the observed FMO activities to FMO3 in the dermis and FMO1 in epidermis and dermis.

The metabolic characterization of RHE helps to identify suitable models for skin toxicity assessment such as sensitization, irritation, and genotoxicityⁱⁱ.

We acknowledge BMBF funding (0315226D)

i: Oesch F, Fabian E, Oesch-Bartlomowicz B, Werner C, Landsiedel R.: Drug-metabolizing enzymes in the skin of man, rat, and pig. *Drug Metab Rev.* 2007;39(4):659-98.

ii: Jackh C, Blatz V, Fabian E, Guth K, van Ravenzwaay B, Reisinger K, Landsiedel R.: Characterization of enzyme activities of Cytochrome P450 enzymes, Flavin-dependent monooxygenases, N-acetyltransferases and UDP-glucuronyltransferases in human reconstructed epidermis and full-thickness skin models. *Toxicol In Vitro.* 2011 Mar 22. [Epub ahead of print]

Comparison of Xenobiotic Metabolizing Enzyme Activities in Normal Human Skin and Reconstructed Human Skin Models from SkinEthic Laboratories

J. Eilstein, G. Léreaux, J.R. Meunier, J. Leclaire and D. Duché

Skin represents the major protective barrier of the body to its environment. Also, skin is an organ involved in the metabolism of xenobiotics and its ability to metabolize xenobiotics can become consequent when considering its total surface area (2 square meters). Consequently, research on skin metabolism would need a real scientific effort to characterize skin metabolizing enzymes and their activities. In addition, the 7th European amendment to the cosmetic directive forbids the use of animal testing to assess the efficacy and safety of new cosmetic ingredients. This policy has forced the cosmetic industry to develop in vitro tools such as reconstructed human skin models (skin model) as alternative methods to animal experiments. For these reasons, these skin models require to be characterized and compared with normal human skin (NHS) samples in terms of metabolic capabilities. This work presents the mRNA expression of several enzymes (CYP450, Esterase, ADH, ALDH, NAT, GST, UGT, SULT...) and their apparent catalytic parameters (apparent K_m , V_{max} and the ratio V_{max}/K_m) in skin models compared with NHS. Results showed that all these enzymes involved in the metabolism of xenobiotics are expressed and effective in the NHS and skin models. Also, apparent ratio V_{max}/K_m (estimating the metabolic clearance) and then the metabolic abilities were often comparable between skin models and NHS. These results indicate that the skin models can substitute to NHS to select cosmetic ingredients on the basis of their metabolism, efficacy or/and safety.

Xenobiotic Metabolism In Human Skin and *in vitro* Alternatives: Phase I & II

Christine Götz, Roland Pfeiffer, Julia Tigges, Ulrike Hübenthal, Eva-Maria Freytag, Hans F Merk, Jean Krutmann, Camilla Pease, Carsten Goebel, Nicola Hewitt and Ellen Fritsche

The metabolic competence of skin has so far not been fully characterized, although human skin fulfills important tasks in uptake, distribution and metabolism of chemicals, such as voluntarily applied substances and anthropogenic pollutants. Since the 7th Amendment to the EU Cosmetics Directive will prohibit the use of animals in cosmetic testing in the coming years, there is an urgent need to understand the xenobiotic detoxifying capacities of human skin and to compare these enzyme activities in *in vitro* models that may be used to replace animals in skin chemical testing. In this work, the enzymatic competences of the most prominent Phase I cytochrome P450 (CYP) and Phase II enzymes were investigated in *ex vivo* human skin, in the 3D epidermal model EpiDerm 200 (EPI-200), immortalized keratinocyte-based cell lines and primary normal human epidermal keratinocytes. Furthermore, cyclooxygenase (COX) activity was assessed, since such enzymes are also capable of phase I oxidation of chemicals through peroxidation. For phase II enzymes, we assessed activities of glutathione S-transferases (GST), N-acetyltransferases (NAT) and UDP-glucuronosyltransferases (UGT) in human skin and the alternative *in vitro* systems. Our data showed that basal Phase I xenobiotic metabolism enzyme activities of reconstructed epidermis are very similar to human skin microsomes. Monolayer cells, however, differ from EPI-200 and from each other in basal and inducible Phase I enzyme activities while they have similar phase II detoxification capacities than human skin. Due to distinct phase I enzyme, especially COX, activities in monolayer compared to 3D organotypic cultures, enzymatic activities of EPI-200 models are closer to human skin than monolayer cultures .

Presentation

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Drivers for Assessing Metabolism of Chemicals in Skin – Safety Assessment for Skin Sensitisation Without Animal Testing

Raniero Zazzeroni - Unilever, UK

Assuring consumer safety associated with the inclusion of new ingredients into products, without the generation of any new animal data on these ingredients, currently poses a considerable scientific and technical challenge. To meet this challenge, Unilever initiated a research programme in 2004, to critically evaluate the feasibility of a new conceptual approach for assuring consumer safety without animal testing.

For many decades, the skin has been known to be a metabolically active organ, yet our understanding of metabolism of chemicals in skin is still limited. Different industries have different drivers for needing to understand skin metabolism. Skin sensitisation is a very important consumer safety endpoint for the cosmetics industry, as many products are either intended for direct application to skin e.g. skin creams and deodorants, or come in to contact indirectly with the skin during use e.g. hair shampoos and conditioners. Many sensitisers are thought to be metabolically activated and as such, better mechanistic knowledge of skin metabolism will be key for consumer safety risk assessment without animal data.

This presentation will give an overview of Unilever's approach to safety assessment for skin sensitisation without animal testing, and why skin metabolism is important in this approach.

ABSTRACT

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Cutaneous Metabolism

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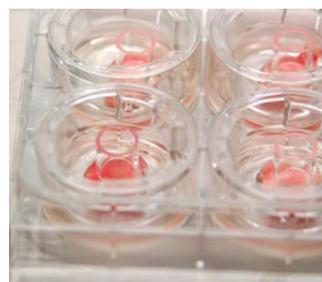
According to the literature, the metabolism of other organs than the liver such the skin seems much less studied. Indeed, skin represents the major protective barrier of the body to the environment and chemicals exposure but is not really yet considered as an organ involved in xenobiotic metabolism. It appears to be a tissue of weak catalytic activity generating less diverse metabolites and less funny reaction mechanisms. However, this assertion could be due to the lack of specific tools to study skin metabolism such as particular sample preparation protocols or analytical methods which are accurate and sensitive enough. Thus, as skin is the largest organ of the human body, even if weak enzymatic activities are observed, they can become consequent when considering its total surface area. Consequently, research on skin metabolism would require a real scientific effort and dynamism to characterize skin metabolizing enzymes and their activities. In addition, the 7th European amendment to the cosmetic directive forbids the use of animal testing to assess the effectiveness and safety of new cosmetics. This policy has forced the cosmetic industry to develop in vitro tools as alternative methods to animal experiments. Reconstructed human skin models are a part of them. For these reasons, these models have to be characterized and compared with normal human skin in terms of metabolic capabilities. This work presents a review of the L'Oréal research strategy and main results in the characterization of skin metabolic equipment and its catalytic capabilities. Thus, characterization for the expression of several enzymes (CYP450, Esterase, NAT, GST, UGT, SULT...) and their catalytic activities (Apparent K_m , V_{max} and clearance) in various reconstructed skins were compared to normal human skin samples.

New Human Skin Organoculture Model to Study Cutaneous Metabolism and Absorption

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BACKGROUND. The methodology for evaluating in vitro percutaneous absorption dates back over 30 years with the innovation of the Franz Cell model (1975). This model has been initially used to study the dermal absorption of specific chemicals. Despite its simplicity, this model is very inconvenient when working with radiolabeled compounds since it implies cells cleaning and radioactive decontamination. The objective of this work was to develop a fast, convenient and easy-to-handle human skin organoculture model for studying both the dermal metabolism and absorption of radiolabeled compounds.

METHODS. Fresh human skin samples (2x2 cm) were seeded dermal side down in polycarbonate Transwell inserts in 6-well plate prefilled with 2 mL culture medium. A 0.5 cm² glass cylinder was placed on the top of the skin sample to match with the upper part usually used in the diffusion cells. To ensure the tightness of the system, skin samples were treated with various volume of Gentian violet and incubations were conducted at 37°C in 5% CO₂ on an orbital shaker in a humidified incubator for 24 hours. Absorbance of Gentian violet in culture medium was measured at 590 nm and lateral leakage of the dye was monitored visually. Histological analysis was performed to check morphological change of skin samples.



RESULTS. Whatever the application volume used, no percutaneous absorption of the dye was measured in the culture medium. Using 5 and 10 μ L application volumes, no lateral diffusion of the gentian violet was observed outside the cylinder while using 20 and 30 μ L application volumes, a lateral diffusion was observed. These results indicate that an application volume of 10 μ L or less is suitable to ensure the tightness of the system. Histological analysis showed a good cytology of the skin and well-preserved overall morphology after 24 hours incubation.

CONCLUSION. This new in vitro model can be adapted to 12- and 24-well Transwell culture plate and can be used for kinetic experiments and for long incubation period. In conclusion, this new in vitro model is a simple, rapid, easy-to-handle and cost effective suitable way to study in vitro dermal absorption and metabolism for radiolabeled as well as cold compounds.

Keywords: Skin organoculture, percutaneous absorption, in vitro metabolism.

Application of a New Human Skin Organoculture Model to Study Cutaneous Metabolism of [4, ¹⁴C]-Testosterone

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BACKGROUND. Dermal metabolism is recognized as an important consideration in evaluating the local exposure of topically applied pharmaceutical products. Different models have been used to study the dermal metabolism of specific chemicals such as Franz cell, skin homogenates and isolated keratinocytes. The objective of this work was to validate the use of a new human skin organoculture model to study skin metabolism using [4, ¹⁴C]-Testosterone as a reference molecule.

METHODS. Fresh human skin samples from two different donors (one man and one woman) were seeded in Transwell inserts in 6-well plate. Skin samples were treated with 10 μ L of [4, ¹⁴C]-Testosterone and incubations were conducted at 37°C in 5% CO₂ on an orbital shaker in a humidified incubator for 4 and 16 hours. After incubation, epidermis, dermis and organoculture medium were extracted and analyzed separately by HPLC with radioactive detection. In vitro skin metabolite profiles were compared with in vitro liver metabolite profiles. Comparison was performed with metabolite profiles obtained in human liver microsomes and cryopreserved hepatocytes.

RESULTS. The results obtained with this new model showed that fresh excised human skin was able to metabolize efficiently [4, ¹⁴C]-Testosterone. Several metabolites were observed after 4 hours and 16 hours in culture medium, epidermis and dermis with no sex related difference. The results also showed that skin metabolism of [4, ¹⁴C]-Testosterone was clearly different from hepatic metabolism. The main metabolites of [4, ¹⁴C]-Testosterone obtained in the skin resulted from reduction reactions whereas the main metabolites obtained in the liver resulted from oxidation reactions.

CONCLUSION. The new human skin organoculture model is an easy-to-handle, simple, rapid and suitable tool to study in vitro cutaneous metabolism of radiolabeled compounds.

Keywords: Skin organoculture, in vitro metabolism, Testosterone.

Metabolic Activities of Reconstructed Human Skin Equivalents and Native Human Skin

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We studied the metabolic capacity of in vitro skin models such as 2D cell systems and 3D reconstructed human skin equivalents (RHEs), the epidermal skin model EpiDerm[®] (MatTek) and the full-thickness skin models EpiDerm[™] FT (MatTek) and Phenion[®] FT (Henkel AG), was investigated and compared to native human skin. Based on previously characterized gene expression profiles enzyme activities for oxidizing enzymes (CYP; FMO) and conjugating enzymes (NAT; UGT) were determined in S9 or microsomal fractions.

CYP 1A, 2B and 3A activities were measured by biotransformation of resorufine derivatives (AROD-Method) as model substrates: Ethoxyresorufine (EROD), Pentoxyresorufine (PROD) and Benzyloxyresorufine (BROD), respectively. FMO 1 and 3 activities were measured by the detection of Benzydamine (BA) biotransformation into BA-N-oxide by HPLC/FLD. The generation of acetylated NAT1 substrate p-Aminobenzoic acid (PABA) was investigated by HPLC/UV. UGT1 activity was determined by detection of glucuronated Methylumbelliferone (MUF) using fluorescence photometry.

CYP activities remained below the detection limit (LOD) of the applied method in all model systems. The limits of detection of the AROD method were 0.002; 0.005 and 0.004 [nmol/min/mg] for EROD, PROD and BROD, respectively. NAT1 activities were determined in RHEs (V_{max} : 11.2 - 16.99 [nmol/min/mg]; K_m = 44 - 53 [μ M]), in human skin (V_{max} : 1.8 \pm 0.7 [nmol/min/mg]; K_m : 35 \pm 10 [μ M]) and in keratinocytes (V_{max} : 0.86 \pm 0.3 [nmol/min/mg]; K_m : 11 \pm 4 [μ M]). The UGT1 activities in the EpiDerm[™] was 1.98 \pm 0.17 [nmol/min/mg], in EpiDerm[™] FT was 0.19 \pm 0.05 [nmol/min/mg], in Phenion[®] FT was 0.16 \pm 0.06 [nmol/min/mg] and in human skin 0.12 \pm 0.01 [nmol/min/mg]. No UGT1 activity in keratinocytes was detectable with the applied method. FMO activities were in the range of 6.68 \pm 1.47 to 3.96 \pm 0.36 [nmol/min/mg] in RHEs or ex vivo human skin while keratinocytes showed no FMO activity.

In summary enzyme activities of RHEs are more similar to native human skin compared to 2D cell systems and should therefore be preferred as in vitro system for toxicological testing in which biotransformation might play a role.

We acknowledge BMBF funding (0315226D).

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