In vitro testing of the penetration and permeation of nanomaterials on an artificial human skin model

M. Weimer, H. Mertsching and H. Brunner

Fraunhofer Institute for
Interfacial Engineering and Biotechnology IGB
Nobelstraße 12, D- 70569 Stuttgart,
weimer@igb.fhg.de
mertsching@igb.fhg.de
brunner@ igb.fhg.de

ABSTRACT

With the help of tissue engineering new products are realized. The purpose of the development is design 3D models with organ-specific characteristics to use them as test systems. With these models it is possible to test effectiveness as well as metabolisation of potential substances as e.g. for nanomaterials. This model can also represent an alternative to animal testing. Thus a possibility of the fast and broad screening is created by pharmaceutical active agents. A condition for such screenings of relevant substances is a standardized and well reproducible *in vitro* system
The purpose of developing such a tissue engineered 3D skin model is to evaluate organ specific influences of materials and nanomaterials on cellular level. Preliminary experiments to analyze the biocompatibility of carbon nanotubes are performed and results will be shown. Further applications of such skin models are analyzing new developed nanomaterials with the aim of considerably reducing or replacing animal testing as demanded by the OECD Test Guideline 428A.
Future emphasis of our work is the establishment of different test systems to investigate wound healing, melanoma research and infection biology.

Keywords: Tissue Engineering, target screening, nanomaterials

INTRODUCTION

Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function. It offers the potential to create replacement structures from biocompatible scaffolds and autologous cells for regenerative medicine.
The living skin equivalent, a 3D organotypic model, has been widely used to investigate many aspects of cutaneous biology. Reconstructed human skin equivalents are models that most closely mimic normal human skin. They allow the topical application and skin irritancy testing of a great variety of products used in daily life. The major requirements for skin penetration screening is the presence of a functional skin barrier. In native skin the first barrier function is carried out by the outside layer of the skin, the *stratum corneum*. The second barrier is the basement membrane. In studies with skin equivalent cultures, it has become evident that these two barrier functions are affected.
Some manufacturers of consumer products, particularly cosmetics, and perhaps in the future food, utilize the advantages derived from including particles in nano size in these products to offer improved or additional functionality. These nanoparticles will be free rather than fixed, although their reactivity (and thus toxicity) may be influenced by coatings. Basically cosmetic products is an area where nanoparticles of oxides of zinc, titanium and iron are being used, and where there are concerns that they might penetrate through the protective layers of the skin and cause reactions with UV light that result in damage to DNA in skin cells. Moreover this corrosion may cause higher irritation potential of cosmetic products or early skin aging.

METHODS

Building of the basic 3D skin model

The development of a skin model requires firstly the isolation and growth of dermal fibroblasts and epidermal keratinocytes from human bioptic tissues. The skin equivalent is generated from primary human keratinocytes on a collagen gel substrate containing human dermal fibroblasts. It is grown at the air-liquid interface which allows full epidermal stratification and epidermal-dermal interactions to occur (Fig. 1). This highly differentiated *in vitro* human skin equivalent model is used to assess the efficacy and mode of action of novel agents.

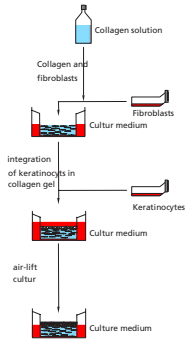

Figure 1: Building of the 3D skin equivalent

Figure 2 demonstrates the natural skin in comparison with the skin equivalent.

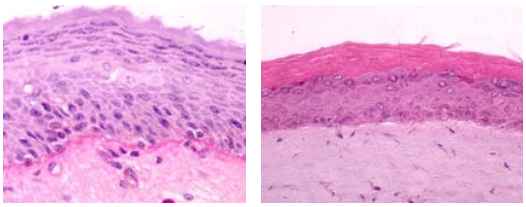

Figure 2: Histological cross section of human skin, and of the 3D skin equivalent with *stratum corneum*

Immunhistochemical staining for cell characterization was performed by use of the avidin-biotin-peroxidase technique. Keratinocytes were characterized by the expression of Keratin 10, 14, 19, Collagen IV, Filaggrin and Involucrin. Streptavidin-peroxidase conjugate was applied, and final staining was performed with diaminobenzidine (Fig 3).

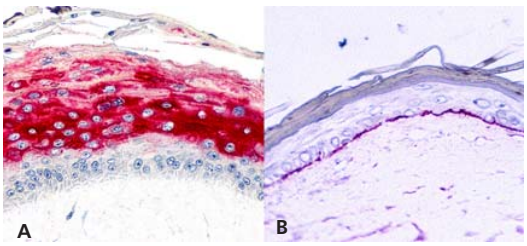

Figure 3: Immunhistological cross section of human skin (A) Cytokeratin 10, (B) collagen IV

RESULTS AND DISCUSSION

We applied our skin equivalent in many different areas such as:

1. **Using the skin equivalent for *in-vitro* toxicity tests**

The irritation effect of different test substances was examined after topical application of the samples on the surface of the skin equivalent (Fig. 4). A cell damage which can be attributed to the substance was photometrical quantified over the reduction from non toxic Tetrazolium salt to water-soluble Formazan [2].

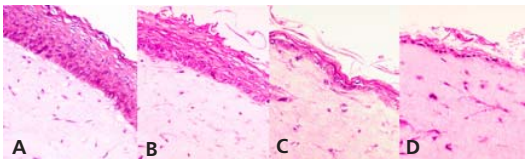

Figure 4: histological cross section:
control (A) after 20% SDS-application for 2 sec (B), 30 sec (C) and 90 sec (D)

2. **Using the skin equivalent as an *in-vitro* tumor model**

The invasion of malignant cells in normal tissues is a fundamental characteristic for progressing and the formation of metastases (Fig. 5). In order to simulate the invasion *in vitro*, different tumor cell lines are co-cultivated. Therefore it is possible to analyze the same influencing variables such as growth factors on the invasion behavior of tumors and to test possible therapeutics (e.g. inhibitors).

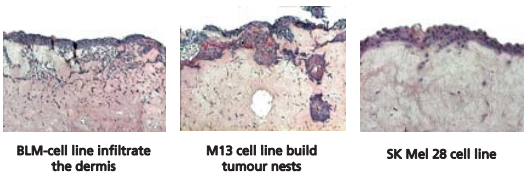

Figure 5: Different aggressive tumor cells on the 3D skin equivalent

3. **Using the skin equivalent for testing *in-vitro* infection**

Infection of the reconstituted skin (A) with a clinical isolate of *C. albicans* (B) and an avirulent strain (C). The clinical strain penetrates the protective layer of keratinocytes and invades through the epithelial cell layers into the matrix, leading to disintegration of the model system after 48 h (B). The avirulent mutants do not form hyphae and show no ability to invade the tissue. *Candida albicans* was only detected on the tissue surface (C). The infection models can also be applied for drug screening [1].

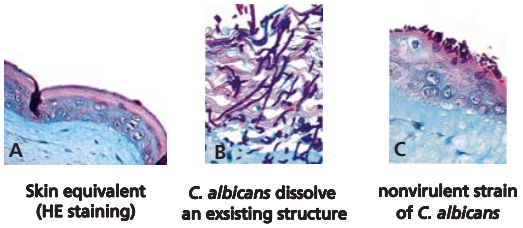

Figure 6: Different strains of *Candida albicans* and their potential to penetrate the skin

4. Using the skin equivalent as an *in-vitro* wound healing model

A wound could be initiated by mechanical effect of an Erb:Yag laser in the artificial skin. The defective region was activated by stimulated keratinocytes of the epidermis to refill the wound (Fig. 7). Parallel the IL-1α expression was measured during the wound healing in the medium by ELISA (Fig. 8). Uninjured skin equivalents served as controls.

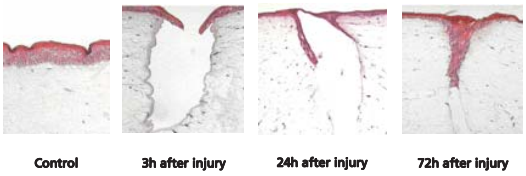

Figure 7: Wound healing process after injury with Laser

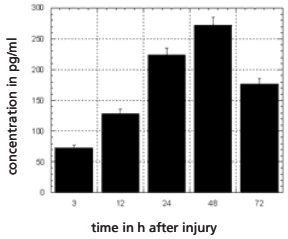

Figure 8: Analysing of Interleukin 1α in the supernatant of the medium during wound healing process.

5 Testing the biocompatibility of carbon nanotubes

The biocompatibility of bucky papers can be carried out using primary cells and the 3D skin equivalent. Figure 9 shows first results with primary human fibroblasts which exhibit improved colonization and proliferation on the surface of specially treated bucky paper.

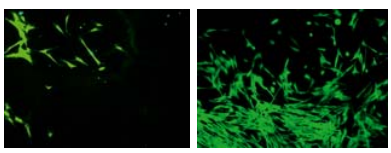

Figure 9: Fluorescence-labeled fibroblasts on bucky paper before and after specific pre-treatment of the paper

By the physiological similarity with natural skin the 3D human skin equivalent is suitable as a test system for:

- determination of the irritation potential of different substances
- pharmacological analysis (e.g. wound healing)
- analysis of infection and invasion of different pathological microorganisms
- target screening
- immunological, histological and molecular-biological analysis
- proof of efficacy and quality control
- penetration- und permeation studies
- development of bio-chips for tumor diagnostics or other skin diseases
- development of medical devices, e.g. laser assisted diagnostic device for melanoma

Pharmaceutical research is hampered by limited predictive value of routinely applied in vitro and in vivo drug screening models for pharmaceutical and clinical efficacy. In drug development, the common approach of pharmaceutical industry is to screen small-molecule libraries for function and toxicity in biochemical based or ligand binding high throughput essays [4]. In general enzymes and 2D cell lines are used in those cell-based assays. The obtained results are of limited biological relevance, since the 2-dimensional cell systems do not adequately mimick the 3D environment in healthy and tumor tissues [5]. This model offers the possibility to simulate physiological drug application and a human 3D test system to established nanomaterials/systems for cancer research/therapy [3].

REFERENCES

[1] Dieterich C, Schandar M, Noll M, Johannes FJ, Brunner H, Graeve T, Rupp S: In vitro reconstructed human epithelia reveal contributions of Candida albicans EFG1 and CPH1 to adhesion and invasion. Microbiology. 2002:497-506

[2]M. Noll, M-L. Merkle, M. Kandsberger, Th. Matthes, H. Fuchs, T. Graeve: Reconstructed Human Skin (AST-2000) as a Tool for Pharmacotoxicology. ATLA, 27, July 1999, 302

[3] Trautmann A, Akdis M, Kleemann D, Altznauer F, Simon HU, Graeve T, Noll M, Brocker EB, Blaser K, Akdis CA: T cell-mediated Fas-induced keratinocyte apoptosis plays a key pathogenetic role in eczematous dermatitis. Clin Invest 2000 Jul;106(1):25-35

[4] Sundberg SA: High-throughput and ultra- high-throughput screening: solution and cell based approaches. Curr Opin Biotechnol, 11, 47-53, 2000.

[5] Balis FM: Evolution of anticancer drug discovery and the role of cell-based screening. J Natl Cancer Inst, 94, 78-79, 2002.